Universidad Autónoma de Madrid

Faculty of Science

Department of Molecular Biology

Doctoral Thesis

Exploring the roles of the SMC5/6 complex in cancer, ageing and kidney disorders

Federica Schiavoni

Madrid 2017

El Dr. Óscar Fernández Capetillo, Investigador Principal del grupo de Inestabilidad Genomica del Centro Nacional de Investigaciones Oncológicas (CNIO), como director.

CERTIFICA

Que Federica Schiavoni, Licenciada en Biotecnología por la Northumbria University, ha realizado bajo nuestra supervisión, la presente Tesis Doctoral “Exploring the roles of the SMC5/6 complex in cancer, ageing and kidney disorders“ y que a su juicio reúne plenamente todos los requisitos necesarios para optar al grado de Doctor/a, a cuyos efectos será presentada en la Universidad Autónoma de Madrid, autorizando su presentación ante el Tribunal Calificador.

Y para que así conste se extiende el presente certificado,

Madrid, 28 de marzo de 2017

Firma del director/es:

Óscar Fernández Capetillo (Director1)

This work has been realized under the supervision of Dr. Óscar Fernández Capetillo, in the laboratory of the genomic Instability group in the Spanish National Cancer Research Centre (CNIO) and was funded by the Innovative Training Networks (ITN) - Marie Skłodowska-Curie Actions.

TABLE OF CONTENT

ABBREVIATIONS

Abbreviations

(6-4 PP)	6–4 photoproduct
4-OHT	4-Hydroxytamoxifen
53BP1	p53-binding protein 1
ADH5	Alcohol dehydrogenase 5
ALDH2	Aldehyde dehydrogenase 2
AML	Acute Myeloid Leukemia
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
a.u.	Arbitrary unit
BER	Base excision repair
BrdU	5-bromo-2′-deoxyuridine
BRCA	Breast Related Cancer Antigens
BLM	Bloom
BS	Bloom syndrome
BTR	BLM-TopoIIIα-RMI1-RMI2 *complex*
CDK	Cyclin dependent kinase
CHK1	Checkpoint kinase 1
CO-FISH	Chromosome Orientation Fluorescence In Situ Hybridization
CPD	cyclobutane pyrimidine dimer
DSB	Double-strand breaks
dsDNA	Double-stranded DNA
DDR	DNA damage response
DEB	Diepoxybutane
FA	Fanconi anemia

FAAP24	Fanconi anemia associated protein 24
FAN1	FANCD2-FANCI associated nuclease 1
HJ	Holliday junction
HR	Homologous recombination
HTM	High-throughput microscopy
HU	Hydroxyurea
ICL	Inter-strand crosslink
IR	Ionizing radiation
IP	Immunoprecipitation
KIN	Karyomegalic interstitial nephritis
NER	Nucleotide excision repair
NPHP	Nephronophthisis
MEFs	Mouse embryonic fibroblasts
MMC	Mitomycin C
MMS	Methyl methanesulfonate
OTA	Ochratoxin A
PI	Propidium iodide
ROS	Reactive oxygen species
RPA	Replication protein A
RS	Replication stress
SCE	Sister chromatid exchange
SMC	Structural maintenance of chromosomes
SS	Seckel Syndrome
ssDNA	Single stranded DNA
SP	Siz1/PIAS

SUMO	Small Ubiquitin-like Modifier
TLS	Translesion synthesis
TopBP1	Topoisomerase II binding protein 1
TOPOII	Topoisomerase II
TOPOIIIα	Topoisomerase III apha
WB	Western blot
WBC	White blood cell count
WRN	Werner
WS	Werner syndrome
UV	Ultra-violet

ABSTRACT

Abstract

The SMC5/6 complex is part of the Structural Maintenance of Chromosomes (SMC) family of proteins, which includes condensin and cohesin and whose main function is the regulation of chromosomal architecture and organization. While it is known that SMC5/6 participates in genome maintenance, how and where the complex operates remains unclear. With the use of mouse models our work has revealed that NSMCE2, a SUMO ligase that is an integral member of the SMC5/6 complex, suppresses cancer and ageing in mice independently of its SUMO ligase activity. At the cellular level, NSMCE2 deficiency leads to severe segregation defects and increased recombination rates, which would be consistent with a role of the SMC5/6 complex in the resolution of joint DNA molecules before chromosome segregation. Besides the overall accelerated ageing that occurs when *Nsmce2* is deleted in adult animals, we here show that these mice present a distinct phenotype on their kidneys that presents all the hallmarks of Karyomegalic interstitial nephritis (KIN). KIN is a progressive kidney disease that in humans has been linked either to exposure to Ochratoxin A, a Topoisomerase II (TOPOII) inhibitor, or to inherit mutations in *FAN1*, an endonuclease mainly associated to the repair of interstrand cross-links (ICL). Our data suggests that NSMCE2 deficiency leads to the accumulation of topological problems, which would ultimately lead to polyploidy and be the cause of the karyomegaly. In support of this, NSMCE2 deficiency sensitizes cells to the inhibition of TOPOII, and leads to a number of phenotypes that are observed upon TOPOII inhibition. In summary, we here present the second mutation that can trigger KIN in mammals, and propose that topological problems, rather than deficiencies in ICLs repair, are the actual cause of this disease.

PRESENTACIÓN

Presentación

El complejo SMC5/6 pertenece a la familia de proteínas SMC (por sus siglas en inglés: Structural Maintenance of Chromosomes), donde también se incluyen cohesina y condensina, y cuya principal función es el control de la organización y estructura de los cromosomas. Si bien se sabe que SMC5/6 participa en el mantenimiento del genoma, cómo y dónde el complejo ejerce su función, no está claro. Utilizando modelos animales, nuestro trabajo ha revelado que NSMCE2, una SUMO ligasa integrante del complejo SMC5/6, suprime el cáncer y el envejecimiento en ratones independientemente de su actividad como SUMO ligasa. A nivel celular la deficiencia en NSMCE2 produce importantes defectos en segregación cromosómica y aumenta la frecuencia de recombinación, lo cual explicaría un papel del complejo SMC5/6 en la resolución de uniones entre moléculas de DNA antes de la segregación. Además del envejecimiento generalizado que ocurre en animales adultos que no expresan NSMCE2, aquí también mostramos que estos animales presentan un fenotipo particular en sus riñones. En concreto poseen los rasgos característicos de la nefritis intersticial cariomegálica o KIN (por sus siglas en inglés: Karyomegalic interstitial nephritis). KIN es una enfermedad progresiva de riñón que en humanos se ha relacionado con la exposición a ocratoxina A, un inhibidor de la Topoisomerasa II, o a mutaciones en *FAN1*, una endonucleasa asociada principalmente con la reparación de uniones cruzadas entre hebras de DNA o “interstrand cross-links” (ICL). Nuestros datos sugieren que la deficiencia en NSMCE2 conduce a una acumulación de problemas topológicos que podrían llevar en último término a la poliploidía y serían la causa de la cariomegalia. Prueba de ello es que la deficiencia en NSMCE2 sensibiliza a las células frente a la inhibición de la Topoisomerasa II y produce fenotipos que se observan tras la inhibición de esta enzima. En resumen, en este trabajo presentamos la segunda mutación que puede desencadenar nefritis intersticial cariomegálica en mamíferos, y proponemos que la causa de la enfermedad es la aparición de problemas topológicos más que las deficiencias en reparación de ICL.

INTRODUCTION

1 - Maintenance of genomic integrity

DNA governs many cellular processes and defines the blueprint of proteins and RNAs within cells. Unlike any other biological macromolecule DNA cannot be replaced. Therefore, errors may have permanent consequences and the inaccurate genetic information will be transmitted to the daughter cells (Garinis *et al*., 2008). Given the importance of preserving genomic integrity, cells have evolved several mechanisms to maintain the fidelity of genetic information, involving sophisticated machineries to repair the lesions, checkpoint pathways and mechanism of DNA damage tolerance (Hoeijmakers, 2009). Failure in these processes often leads to pathological conditions such as premature ageing, a variety of inherited diseases and predisposition to cancer (Aguilera and Gómez-González, 2008).

1.1 - Sources of DNA damage and the DNA damage response (DDR)

Around 1000 single-strand breaks and base losses are estimated to occur daily in every cell. The total amount of lesions per cell can rise up to 100,000 lesions per day considering other types of DNA damages that may occur (Garinis *et al*., 2008, Lindahl, 1993). Insults to genomic integrity can arise from exogenous agents but also from the products of endogenous metabolism **(Figure 1a)**. The most frequent source of exogenous DNA damage is ultraviolet light (UV). UV-A and UV-B light are not filtered by the ozone layer and they can induce a conspicuous number of photoproducts. 100,000 lesions per cell are estimated to occur after one hour exposure to strong sunlight (Jackson and Bartek, 2009). DNA integrity is also threatened by the exposure to ionizing radiation (IR), which can induce a range of lesions, including DNA double strand breaks (DSBs), which are the most toxic of all (Jackson and Bartek, 2009).

Normal cellular metabolism is also a potential DNA damaging source. Although it is required to maintain fundamental biological processes, it can contribute to genomic instability in various ways. For instance, internal metabolism produces reactive oxygen species (ROS) deriving from oxidative respiration and lipid peroxidation, which are radical species that can attack DNA (Jackson and Bartek, 2009). Given the dangerous presence of these compounds within the nucleus, it is not surprising that evolution has provided a series of ROS caretaking systems to overcome the price of cell own metabolism (Hoeijmakers, 2001). Besides ROS, recent findings indicate that the endogenous production of aldehydes can also promote crosslinks in both DNA and proteins. For instance, endogenous formaldehyde is abundant and ubiquitous within cells. This compound can be produced directly from histone demethylation reactions that take place on chromatin, thus posing a direct challenge to

genomic integrity (Pontel *et al*., 2015, Stingele *et al*., 2016, Rosado *et al*., 2011). DNA can also undergo spontaneous chemical deterioration, thereby, creating abasic sites and deamination. The deamination of cytosine, adenine and 5-methylcytosine can convert these bases into a miscoding base (e.g. uracil) (Hoeijmakers, 2001).

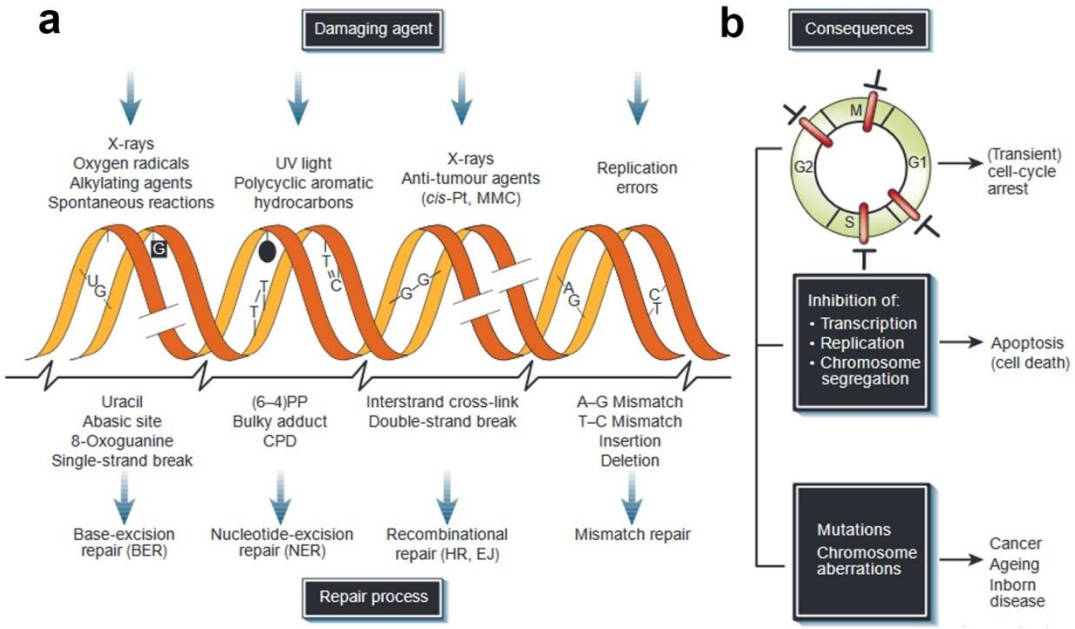

Fig. 1 - Sources and repair of DNA damage (Hoeijmakers, 2001)

a) Common endogenous and exogenous sources of DNA damage and specific repair of such lesions. X-rays, oxygen radicals, alkylating agents and spontaneous reactions can induce types of DNA damage (such as uracil, abasic site, 8-Oxoguanine, ssDNA) that are repaired through the Base Excision Repair pathway (BER). UV light and polycyclic aromatic carbons can generate 6-4 photoproduct (6-4 PP), cyclobutane pyrimidine dimer (CPD) and bulky adduct that are repaired by the Nucleotide-Excision repair pathway (NER). Exposure to X-ray and a certain class of chemotherapeutic drugs (Cisplatin and Mitomycin C) can instead lead to Inter-strand crosslink lesions and DSBs that are dealt by the Homologous recombination (HR) pathway. In addition, errors introduced by the replication machinery are repaired by the mismatch repair pathway.

b) Possible outcome following the activation of the DNA damage response (DDR). Activation of DDR may induce cell cycle arrest in order to provide time for the repair of the lesion. Eventually, the apotosis pathway can be activated. When changes in the DNA sequence are not repaired, they persist in the genome and can trigger cancer and ageing.

The consequences of the damage can greatly vary depending on the type, the severity of the injury and its localization within the genome. Besides generating point mutations, the hazard posed by DNA injuries comes from the potential perturbation of chromatin transactions such as transcription and replication. Regarding transcription, lesions can block the transcription process, either inactivating the transcribed gene or affecting the transcribed product. When

interfering with DNA replication, fork stalling can lead to the breakage of the fork, with the subsequent risk of genomic rearrangements.

In order to protect genetic information from genotoxic insults, cells have evolved an interwoven and sophisticated DNA repair system. The maintenance of genomic integrity includes multiple multistep DNA repair pathways, covering most of the insults that can be inflicted to a cell. Most of these repair programs are coordinated though a common signal transduction pathway activated by DNA damage and replication stress, and overall known as the DNA damage response (DDR) (Harper and Elledge, 2007). Upon activation of the DDR, the cell cycle is halted at its various checkpoints (primarily G1 and G2), which arrest the cell cycle in order to provide time to repair the lesion. If the damage is too severe, cells may instead choose to activate apoptosis, leading to cell death in a tightly regulated manner **(Figure 1b)** (Hoeijmakers, 2001).

1.2 - Replication stress is a major threat to genomic integrity

In addition to the spontaneous vulnerability of DNA and the exposure to exogenous and endogenous damaging compounds, major injuries can arise during DNA replication itself, thus posing at risk the genomic integrity in each cell cycle (Lecona and Fernández-Capetillo, 2014). The variety of events challenging normal fork progression is known as Replication Stress (RS).

The replisome has to face numerous obstacles, such as DNA modifications, difficult to replicate structures or limiting amounts of the nucleotide pool. All these challenges may lead to replication fork stalling and accumulation of recombinogenic single stranded DNA (ssDNA). Although most of the ssDNA is generated in S-phase, accumulation of ssDNA can also occur after DSB resection or at patches generated by double-nicks during Nucleotide excision Repair (NER). ssDNA becomes rapidly coated by the ssDNA binding protein RPA (Replication protein A). These structures are recognised by the kinase ATR, which is then recruited to the lesion by binding to RPA through the ATR interacting protein ATRIP **(Figure 2)** (López-Contreras and Fernandez-Capetillo, 2010). At the arrested forks, ATR is activated allosterically by interaction with factors such as TopBP1 or ETAA1. Once active, ATR phosphorylates multiple factors including the key checkpoint protein CHK1 (Checkpoint kinase 1). CHK1 lowers CDK activity thereby preventing the entry into mitosis when DNA has not been fully replicated (Lecona and Fernández-Capetillo, 2014, López-Contreras and Fernandez-Capetillo, 2010). A failure in this cascade would lead to the presence of unreplicated loci in mitosis, leading to major segregation problems and ultimately cell death.

Accordingly, death by ATR inhibitors can be rescued by preventing the premature mitotic entry induced by these chemicals (Ruiz *et al*., 2016).

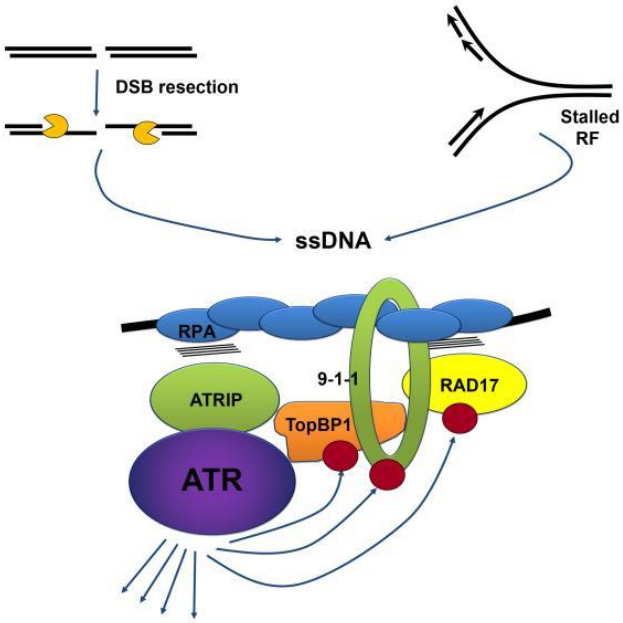

Fig. 2 - Activation of the ATR pathway (López-Contreras and Fernandez-Capetillo, 2010).

RPA-coated ssDNA, generated from DSB resection and stalled replication forks, recruits the kinase ATR to the site of damage. RPA also recruits Rad17 that is responsible for the loading of PCNA-like heterodimeric ring 9-1-1. This complex then activates ATR by allowing the interaction of the kinase with TopBP1.

In any case, targeting ATR activity has shown to have potential as an anticancer strategy. The idea behind this therapy is that limited ATR activity should be particularly toxic for cells presenting high endogenous levels of RS, such as those carting various oncogenic mutations (Halazonetis *et al*., 2008). In support of this view, low levels of ATR prevent the development of Burkitt lymphomas induced by MYC in mice (Murga *et al*., 2011) and ATR inhibitors have already showed efficiency in preclinical models of various tumors with high levels of RS such as Ewing sarcomas and Acute Myeloid Leukemia (AML) (Nieto-Soler *et al*., 2016, Morgado-Palacin *et al*., 2016).

1.3 - Homologous recombination-mediated repair and processing of Holliday junctions

DSBs represent the most dangerous threat to DNA integrity. This is mostly due to the difficulties to repair DSBs compared to other types of DNA damage. In addition, erroneous rejoining of the broken ends may result in severe alteration of the genetic information, due to loss or amplification of chromosomal regions and translocations (Khanna and Jackson, 2001). DSBs can occur as a consequence of RS when the replication fork encounters a blocking lesion leading to fork collapse. Otherwise, DSBs can arise from the exposure to IR and from certain DNA damaging compounds. Moreover, immune cells and gametes use

DSB-intermediates as part of their recombinatorial processes (Shrivastav *et al*., 2008, Khanna and Jackson, 2001).

DSBs are preferentially repaired by Homologous Recombination (HR), a highly complex and precise DNA repair pathway that uses the information of the complementary sequence in the other duplex to drive the repair of the broken chromosome (Mimitou and Symington, 2009). Since HR uses the replicated chromatin as a template (rather than the homologous chromosome to prevent loss of heterozygosity) it is restricted to S and G2 phases of the cell cycle. The initial step involves the degradation of the dsDNA by nucleases to generate 3′ ssDNA tails **(Figure 3)**, which are rapidly protected by RPA coating. Next, RAD51 assembles onto the ssDNA to form a nucleoprotein filament with the assistance of mediator proteins such as BRCA2. The RAD51 filament then searches the homologous sequence and promotes the invasion of the ssDNA into the homologous targeted region to form a joint molecule. After RAD51 removal, DNA polymerases synthesize the missing region by using the homologous chromatid as template (Forget and Kowalczykowski, 2010, Mimitou and Symington, 2009).

The switching of strands between chromatids leads to the formation of a cross-stranded structure, known as Holliday junction (HJ), that physically links the two recombining DNA molecules by a four-way junction (Matos and West, 2014). The physical evidence of recombination intermediates was provided for the first time by electron microscopy studies conducted in the early 1970 (Doniger *et al*., 1973, Thompson *et al*., 1975, Benbow *et al*., 1975, Liu and West, 2004). The disentanglement of HJs can lead to crossover or non-crossover products depending on the pathway that is selected for the repair **(Figure 3)**.

In somatic cells, HJs are preferentially processed through the dissolution pathway, which separates chromatids without the formation of crossover products. The dissolution of the linked DNA molecule is achieved by the BTR complex, composed of the helicase BLM, the topoisomerase III alpha (TOPOIIIα) and the RecQ-mediated instability factors, RMI1 and RMI2. BLM catalyses the migration of a double HJ towards one another, collapsing it into a hemicatenane, that is then cleaved and religated by TOPOIIIα (Swuec and Costa, 2014, Cejka *et al*., 2010).

If disentanglement cannot be accomplished through the anti-recombinogenic pathway of dissolution, a nuclease-dependent pathway, known as resolution, takes over to cleave the linked chromatids (Mankouri *et al*., 2011). Nucleolytic resolution is triggered by CDK1-dependent phosphorylation of SLX4, which acts as a platform for the activity of the MUS81/EME1 nuclease complex. If all of the above fail and junctions persist, the last attempt to resolve these intermediates relies on the GEN1 nuclease. In contrast to MUS81, which is

activated in mitosis through phosphorylation, the premature action of GEN1 is limited by excluding it from the nucleus until the nuclear envelope breaks down during mitosis (Hustedt and Durocher, 2016, West *et al*., 2015, Chan and West, 2014, Garner *et al*., 2013). All of these nucleases resolve HJs by introducing symmetrical nicks in strands with the same polarity, upon which the broken DNA can be simply religated. Again, whether a crossover or non crossover product is generated during this reaction depends on the orientation of the cleavage (Matos and West, 2014).

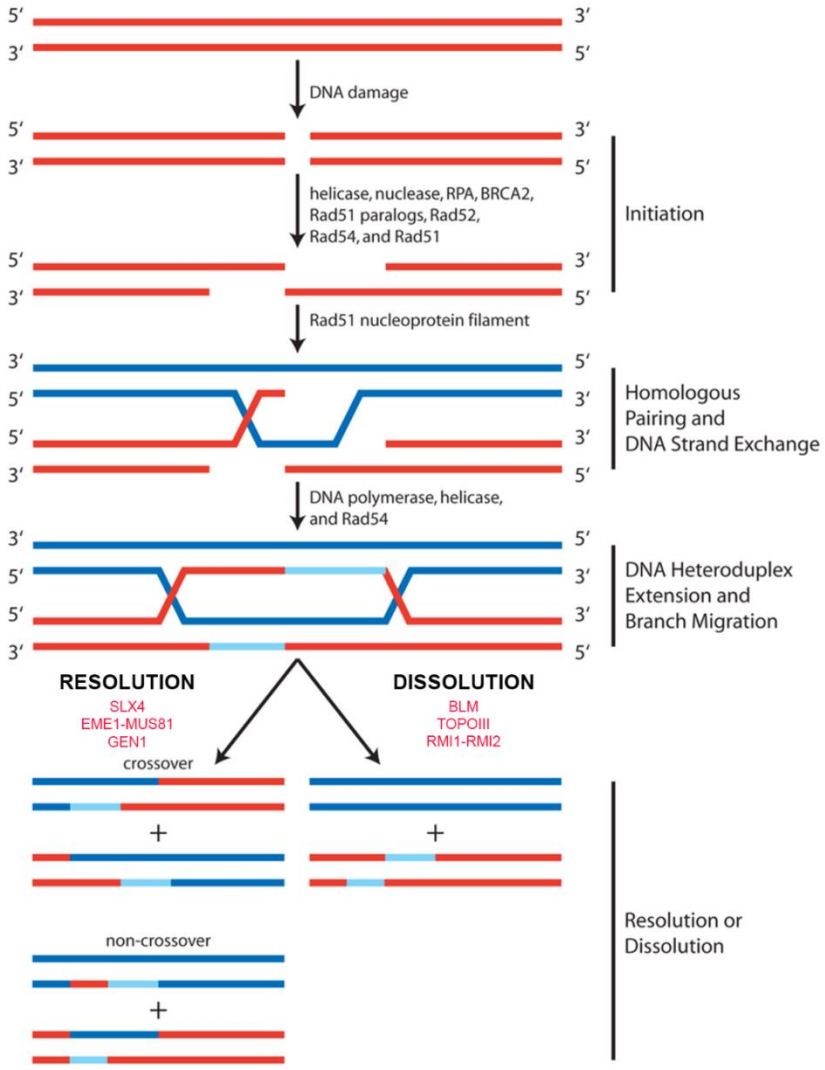

Fig. 3 - HR-mediated repair leading to the generation of a double Holliday junction that is processed either by the anti-recombinogenic pathway of dissolution or by the nuclease-dependent pathway of resolution. Modified from Forget and Kowalczykowski (2010).

The repair of DSBs starts with the resection of the 5′ terminated strand, which results in a 3′ terminated ssDNA tail. Rad51, assisted by several factors such as BRCA1, assembles as nucleoprotein filament onto ssDNA. Next, Rad51 locates the homologous region on the homologous chromosome and exchanges DNA strands. The synthesis of the missing DNA leads to the formation of two HJs. These structures will be either processed through the dissolution pathway by BTR complex (BLM, TOPOIIIα, RMI1 and RMI2) without the generation of crossover products, or by the recombinogenic pathway of resolution, that is achieved by nucleases and allows the exchange of genetic material between homologous chromosomes.

1.4 - Consequences of replication stress in mitosis

If checkpoints fail to block mitotic entry, the presence of non-fully replicated regions or unresolved repair intermediates can have severe consequences on chromosome segregation (Mankouri *et al*., 2013). There are a number of sources of problems that can limit DNA replication. For instance, HR-mediated repair involves the formation of joint DNA molecules at the HJ (Chan and West, 2015). If these structures persist at mitosis, the physical association between sister-chromatids would impede their disjunction in anaphase, resulting in DNA bridges **(Figure 4)** (Gelot *et al*., 2015, Hustedt and Durocher, 2016). Due to their toxicity, mitotic cells have several non-redundant pathways in order to process such structures and allow chromosome segregation.

The presence of unreplicated DNA constitutes a potential source of DNA breaks. The mechanical tension generated in anaphase can lead to the breakage of the unreplicated chromosome, resulting in the uneven segregation of the broken chromosome arms (Magdalou *et al*., 2014). Alternatively, if the tension fails to pull the two chromatids apart both chromatids may be pulled towards the same mitotic spindle, leading to aneuploidy (Gelot *et al.*, 2015). Moreover, unresolved replication is frequently associated with the generation of extra centrosomes in mitosis, which are often functional and can promote multipolar mitosis and unbalanced segregation (Magdalou *et al*., 2014, Gelot *et al*., 2015). Mis-segregated or broken chromosome pieces are thought to be the source of micronuclei. Also, the signal of the unresolved DNA damage can be transmitted to the next G1 phase and can be visualized as accumulation of the protein 53BP1, known as 53BP1 bodies. Their function is still unclear, but it is likely that these structures are necessary to mark and facilitate the repair of these lesions, or to block their repair until S phase when HR becomes available (Gelot *et al*., 2015, Hustedt and Durocher, 2016, Lukas *et al*., 2011).

In some circumstances cells can bypass mitosis, although having replicated their genome in the previous S-phase. Therefore, the cell will start the new cycle with an altered ploidy status, carrying double amount of DNA. While polyploid genomes are common in plants and the progressive accumulation of polyploid cells is physiological in some tissues, the duplication of the genetic material has to be rather considered a pathological event. Changes in the genome content are believed to contribute to genomic instability and to provide a route to tumorigenesis. Several mechanisms have been identified in promoting polyploidization, including cell fusion and a variety of defects that interfere with the cell cycle machinery (Storchova and Pellman, 2004). Noteworthy, the presence of unresolvable and/or unreplicated chromosomes can also promote mitotic slippage. Hence, replication stress can also be a source of alterations in ploidy such as aneuploidy or poliploidy.

2 - Physiological outcome of defective DNA damage response

2.1 - Genomic instability can trigger cancer and ageing

In response to persistent genomic insults, the DDR can induce apoptosis or cellular senescence, a condition of permanent growth arrest that represents a physiological mechanism to counteract tumorigenesis (Bartek *et al*., 2007, Maslov and Vijg, 2009). In agreement with this, cancer cells often acquire mutations conferring the ability to disable the DDR barrier, thus, allowing to escape apoptosis and senescence. These cells will accumulate further mutations and give rise to neoplastic lesions (Campisi, 2003). Although cellular senescence was always associated with anti-tumorigenesis, recent findings suggests that senescence is a 'double edge sword' as it can also drive malignant transformation. Senescence cells often secrete degradative enzymes, growth factors and cytokines, which may alter the homeostasis of the tissue microenvironment and promote proliferation and malignant phenotypes in neighbour cells (Magdalou *et al*., 2014, Campisi, 2003). Finally, and besides its role in cancer, apoptosis and cellular senescence can compromise the functionality of a certain tissue and can impair the regenerative potential of stem cells, both hallmarks of ageing (Collado *et al.*, 2007). Likewise, the accumulation of senescence within tissues is associated with the development of age-related pathologies (Campisi, 2003).

2.2 - Defective DNA repair syndromes

Deficiencies in DNA repair are a frequent source of hereditary diseases. For their study, mouse models have provided a unique tool to investigate on their molecular basis and have provided valuable insights into the consequences of genomic instability *in vivo*. Some of these disease models are cancer prone, whereas some defects are associated with a premature ageing phenotype. However, in both cases there is an enormous variability of clinical signs. Although a clear link between defects and phenotypes has not been provided yet, it is very likely that the effect of genomic instability is mostly dependent on the severity of the mutation. While severe deficiencies in the DDR pathways are more likely to promote senescence and or cell death, and therefore lead to ageing, milder conditions may increase the mutation burden and thus predispose to cancer (Finkel *et al.*, 2007).

2.2.1 - Premature ageing syndromes

Progeroid syndromes support the tight association between the accumulation of DNA damage and ageing. In fact, most premature ageing diseases have been linked to defects in

DNA repair. One example, which is linked to replication stress, is that of the Seckel Syndrome (SS). SS is an autosomal recessive disorder. It has been described for the first time in the early sixties by Helmut Seckel (Seckel, 1960) and it is characterized by intrauterine growth retardation, severe dwarfism, microcephaly and mental retardation (O'Driscoll *et al*., 2003). In one of its forms, the disease in humans derives from a mutation in the ATR gene, which introduces a splicing defect and reduces significantly the amount of ATR. Yet a residual level of protein is still sufficient to allow compatibility with life (Murga *et al*., 2009, O'Driscoll *et al*., 2003). These individuals present impaired ATR activity that plays a critical role in sensing replication stress. ATR induces a signalling pathway that leads to cell cycle arrest and eventually to the repair of replicative damage. A SS mouse model generated in our lab (Murga *et al*., 2009) recapitulates the phenotypes observed in humans to a large extent. Beside dwarfism and developmental defects, these mice died prematurely (within the first 6 months), and presented several phenotypes associated with ageing, including hair greying and decreased density of hair follicles, thinner epidermis, osteoporosis, accumulation of fat in the bone marrow and pancytopenia.

Perhaps the best example of premature ageing syndromes is the Werner syndrome (WS), due to its remarkable similarity to normal ageing (Ciccia and Elledge, 2010). 58 mutations in the REQ-like helicase *WRN* have been identified in patients with WS phenotypes. All these mutations result in the expression of a truncated protein and in the loss of the helicase and exonuclease activities of WRN (Kudlow *et al*., 2007, Huang *et al*., 2006, Friedrich *et al*., 2010). Individuals affected by WS develop normally until the first decade of life. Onset of symptoms usually occurs in the early 20s, when patients start to display a variety of ageing phenotypes such as short stature, bilateral cataracts, early greying and loss of hair, type 2 diabetes mellitus, osteoporosis and scleroderma-like skin changes (Huang *et al*., 2006, Kudlow *et al*., 2007). Beside features of accelerating ageing, these individuals are also highly predisposed to develop cancer. The median age of death has been reported to occur between 47-54 years, usually as a result of malignancies or cardiovascular diseases (Rossi *et al*., 2010, Huang *et al*., 2006). As in the case of ATR, the role of the WRN helicase has been linked to the processing of DNA substrates generated during HR repair and in telomere maintenance. In absence of WRN these toxic intermediates and short telomeres accumulate, triggering genetic instability, DNA damage response and apoptosis (Kudlow *et al*., 2007). Given that WRN has a critical role in multiple pathways, it is not surprising that WRN deficiencies result in such a severe phenotype.

In regards to mouse models, one of the most studied model for ageing is the ERCC1-deficient mouse model. ERCC1 forms a specific endonuclease complex by association with the protein XPF. The ERCC1/XPF complex catalyses the initial incision step in the

Nucleotide Excision repair (NER) pathways after a variety of DNA insults, including UV adducts and inter-strand crosslink (ICL) (Gregg *et al.*, 2011). Generation of ERCC1-deficient mice was first reported by two independent studies (McWhir *et al*., 1993, Weeda *et al*., 1997). McWhir *et al*. (1993) targeted exon 5 resulting in the expression of a truncated protein lacking of the XPF-interacting domain. The second transgenic strain was generated by Weeda *et al*. (1997) by inserting a neomycin resistant cassette into *Ercc1* exon 7, also affecting the interaction domain with XPF (Gregg *et al*., 2011). Both models suffer from severe growth impairment: the knockout mice weight only 20% of control animals as well as mice display reduced lifespan, dying prematurely at the age of three weeks. Although the hematopoietic system is initially normal, mice become rapidly leukopenic and thrombocytopenic and adipose tissue gradually accumulates in the bone marrow (Gregg *et al*., 2011). Interestingly, like many DNA repair pathologies, ERCC1 deficiency affects preferentially one specific organ, where ERCC1 is likely to fulfil a more crucial function. This phenomenon is normally known as segmental progeria. For instance, major abnormalities in liver nuclei emerged by histological examination in ERCC1-deficient mice including an accumulation of polyploid and aneuploid hepatocytes (Gregg *et al*., 2011, McWhir *et al*., 1993, Weeda *et al*., 1997). Such alteration ultimately leads to an impaired liver function as demonstrated by the increase of liver enzymes in serum (McWhir *et al*., 1993). Similar abnormalities were also found in other tissues such as skin, spleen and kidneys (Weeda *et al*., 1997). Defects in ERCC1 in humans cause celebro-oculo-facio-skeletal (COFS) syndrome, which is characterised by a severe growth retardation, microcephaly, facial dysmorphism, kyphosis and psychomotor disability (O'Driscoll *et al*., 2003).

2.2.2 - Cancer-prone syndromes

For some defects in DDR genes, there is a very strong association with cancer predisposition. For instance, a number of tumour suppressor genes are indeed involved in DNA repair and their loss results in severe genomic instability. Again, inherited mutations in these gene also leads to severe hereditary disorders.

The Bloom syndrome (BS) is one of the best-characterised cancer-prone syndromes. The disease can be caused by different mutations in the *BLM* gene, which encodes the RecQ helicase BLM (Carrero *et al*., 2016). Although WRN and BLM helicases belong to the same family of proteins, mutations in these genes result in very distinct clinical outcomes. While BS affects significantly the lifespan without any evident sign of accelerated ageing, onset of ageing-associated features occurs prematurely in individuals affected by WS (de Renty and Ellis, 2017). BS patients generally die before the age of 30 due to complications from cancer.

It was estimated that affected individuals are 99 times more likely to be diagnosed with cancer compared to the general population (de Renty and Ellis, 2017). Interestingly, loss of functional BLM can predispose to cancers of different cellular origins and many cancer types have been reported to be associated with such mutations (Hickson, 2003).

High incidence of malignancies in BS patients is a result of the hyper-recombination displayed by BLM-deficient cells, which is the distinctive hallmark of this genetic disorder **(Figure 5)**. Loss of BLM results in a 10-fold increase in Sister Chromatid Exchange (SCE), which derives from the repair of DNA joint molecules (de Renty and Ellis, 2017). The helicase BLM is implicated in a recombination free pathway that ensures that replication intermediates are processed without crossover (Ira *et al*., 2003). In cells isolated from BS patients these structures are instead processed through the resolution pathway that allows the exchange of genetic material between sister-chromatids, leading to a major increase in sister chromatin exchange events.

Fig. 5 - Hyper-recombination in BLM-deficient cells (Heyer, 2004).

Exchange of genetic material between sister-chromatids in BLM-deficient cells isolated from a patient affected by BS. Chromatids are labeled with two different dyes in order to visualize exchange between sister-chromatids.

Along with the recombination phenotype, BLM deficiency causes a variety of chromosomal defects at cellular level, including chromosome breaks, telomere associations, radials, lagging chromosomes and anaphases bridges (de Renty and Ellis, 2017, German *et al*., 1974).

Similarly to BS, Fanconi anaemia (FA) is also a genetic condition characterized by genomic instability and predisposition to cancer. In this case, the disease is triggered by defects in the DNA repair pathway that deals with ICLs, one of the most deleterious insult that can be inflicted to DNA. Therefore, FA has been extensively studied as a disease model in order to give insights on how repair of ICLs is achieved (Deans and West, 2011). Up to now, mutations in 19 genes have been linked to FA and for some patients the genetic defect responsible for the disease has not been identified yet (Kottemann and Smogorzewska, 2013). All these proteins are believed to operate in a common multistep cellular pathway that coordinates several repair systems, including HR, nucleotide excision repair (NER) and translesion synthesis (TLS) (Deans and West, 2011).

One of the current models proposes that the ICL lesion is first recognized by FANCM, a DNA binding protein with helicase motifs, together with Fanconi anaemia associated protein 24 (FAAP24) and the histone fold proteins MHF1 and MHF2. Once on chromatin, FANCM acts as a platform for the assembly of the FA core complex, which consists of 14 proteins (**FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM**, FANCT, **FAAP100,** MHF1, MHF2, FAAP20 and FAAP24). Upon activation, the ubiquitin ligase FANCL, which is part of the FA core complex, promotes the mono-ubiquitination of FANCD2-FANCI heterodimer (Ceccaldi *et al.*, 2016, Wang, 2008). Ultimately, mono-ubiquitinated FANCD2-FANCI recruits downstream effectors of the FA pathway, including SLX4 which activates structure-specific endonucleases such as ERCC1, ERCC4, MUS81-EME1 and SLX1. The nucleolytic incision generates DSBs that are finally repaired by HR **(Figure 6)** (Ceccaldi *et al*., 2016).

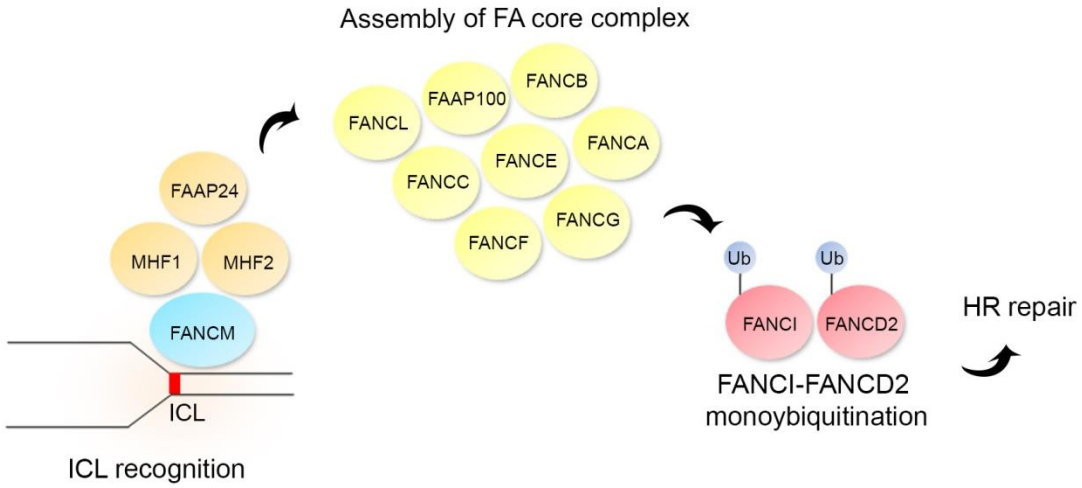

Fig. 6 - Activation of the FA pathway.

The ICL lesion is sensed by FANCM. Upon activation, FANCM induces the formation of the FA core complex, which is composed by 14 FA proteins. The ubiquitin-ligase FANCL, within the FA core complex, mono-ubiquitinates and activates the FANCD2-FANCI heterodimer. Next, the FANCD2-FANCI heterodimer recruits nucleases to excise the lesion. DSBs arised from the nucleolytic cleavage are processed by the HR pathway.

At a cellular level, the FA patients accumulate DNA damage at increased rates and cells from affected individuals are hypersensitive to ICL agents, such as Mitomycin C (MMC) and diepoxybutane (DEB). Upon ICL damage, radial chromosomes and breaks characterize the metaphase spreads of FA-deficient cells **(Figure 7a)** and the quantification of these aberrations are commonly used in the clinic to provide a diagnosis of FA (Moldovan and D'Andrea, 2009).

Another striking feature of FA is the bone marrow failure, indicating the crucial role of the FA pathway in hematopoietic stem cells expansion and maintenance (Ceccaldi *et al*., 2016,

Kottemann and Smogorzewska, 2013). The onset of haematological defects occurs within the first two decades of life and they include anemia **(Figure 7b)** and thrombocytopaenia, which are frequently proceeded by neutropaenia. The affected individuals often develop clonal abnormalities in the bone marrow, which may lead to myelodysplasia or AML (D'Andrea and Grompe, 2003). FA patients can develop a variety of cancer types besides AML, including gynaecological, head and neck squamous cell carcinoma, oesophageal cancer, liver, brain, skin and renal tumours (Michl *et al*., 2016). Due to hypersensitivity of patients cells to ICL-inducing agents, these compounds are excluded from treatment strategies of FA cancer patients (Deans and West, 2011), which severely limits their choices for treatment.

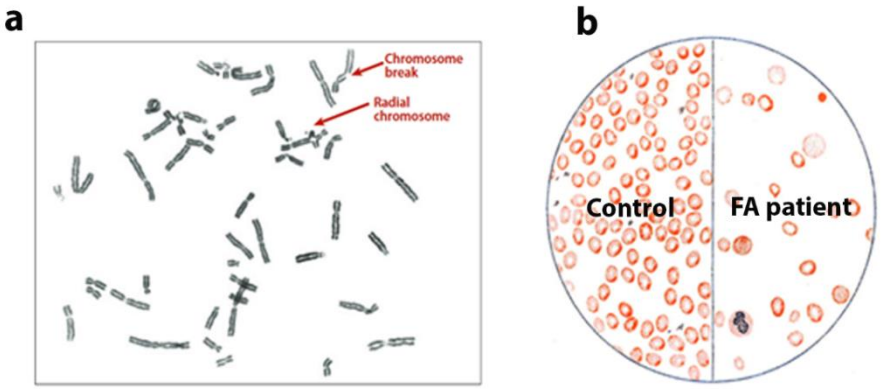

Fig. 7 - Hallmarks of Fanconi anemia.

a) Presence of radial chromosomes and chromosome breaks in metaphase spread of cells isolated from FA patients (Moldovan and D'Andrea, 2009).

b) Analysis of erythrocytes performed by Guido Fanconi on the first FA patient in 1927. Erythrocytes from control (left) and FA patient (right). FA blood sample shows few, large and irregularly shaped erythrocytes. Modified from Lobiz and Velleuer (2006).

Although the FA pathway repairs lesions originated from ICL-inducing chemotherapeutic agents, FA patients do not usually come in contact with these compounds during their lives. It has thus been postulated that the genotoxic threat can instead arise from endogenous metabolism. Namely, that the clinical manifestations of the FA syndrome are due to the accumulation of endogenous damage that cannot be processed when the FA pathway is defective. In support of this hypothesis, sensitivity to formaldehyde and acetaldehyde, which are generated in cells as byproducts of our own metabolism, has been shown in cells mutated for different FA genes (Rosado *et al*., 2011). Moreover, inactivation of the FA pathway in combination with a deficiency in alcohol dehydrogenase 5 (ADH5), the main enzyme in formaldehyde catabolism, results in synthetic lethality in DT40 cells (Rosado *et al*., 2011). A double knockout for FANCD2 and aldehyde dehydrogenase 2 (ALDH2),

essential for the elimination of toxic acetaldehyde, was also synthetic lethal in mice (Langevin *et al*., 2011, Garaycoechea *et al*., 2012). All these studies support the contribution of the FA pathway in the repair of acetaldehyde and formaldehyde-induced damage, and prove that the FA pathway becomes indispensable in absence of enzymes able to catabolise these compounds.

2.3 - DNA damage and kidneys disorders

Finally, and besides cancer and ageing, a connection between DNA damage and kidney disorders emerged recently from clinical cases and from transgenic mice work. These studies demonstrated that particular types of DNA damage, not yet fully understood, can lead to a distinct pathological condition known as Karyomegalic Interstitial Nephritis (KIN). KIN is a rare nephronophthisis (NPHP)-related condition characterized by renal tubular degeneration that progressively leads to kidney failure. Diagnosis of KIN occurs through histological analysis that reveals the striking features of KIN: tubular basement membrane degeneration, tubular microcysts and dilation, widespread fibrosis and tubular cell karyomegaly (enlarged nuclei) **(Figure 8a)** (Zhou *et al*., 2012, Lachaud *et al*., 2016). The enlargement of tubular cell nuclei reflects the ploidy status of these cells. The use of probes hybridizing to selected chromosomal regions confirmed the polyploidy content of kidney cells in transgenic mice affected by KIN (Thongthip *et al*., 2016).

Up to now, only mutations in the *FAN1* gene have been reported to induce KIN in humans (Zhou *et al*., 2012). The nuclease FAN1 was first identified through genetic screenings designed to identify factors providing resistance to ICL damage (Zhou *et al*., 2012, Smogorzewska *et al*., 2010). Driven by the clear implication of the nuclease in ICL repair, many studies investigated the connection of FAN1 with the FA pathway that is the main route to repair ICLs. In fact, FAN1 was shown to interact with FANCD2 and this interaction is critical for FAN1 localization at the site of damage (Smogorzewska *et al*., 2010, MacKay *et al*., 2010). These observations placed FAN1 as a key downstream effector of the FA pathway and provided evidence of KIN being a condition triggered by defects in the repair of ICLs. Also, FAN1-deficient cells isolated from patients are sensitive to ICLs and display radial chromosomes in their metaphases upon damaging conditions, both common hallmarks of FA (Zhou *et al*., 2012) **(Figure 8b)**.

Despite the phenotype similarity and the collaboration of FAN1 with the FA pathway, individuals carrying mutations in *FAN1* gene do not suffer from all the clinical signs displayed by FA patients, such as cancer predisposition and bone marrow failure. Therefore, *FAN1* is

not a typical susceptibility gene for FA and might have a distinct role in DNA repair, which cannot be restricted to the FA pathway (Lans and Hoeijmakers, 2012).

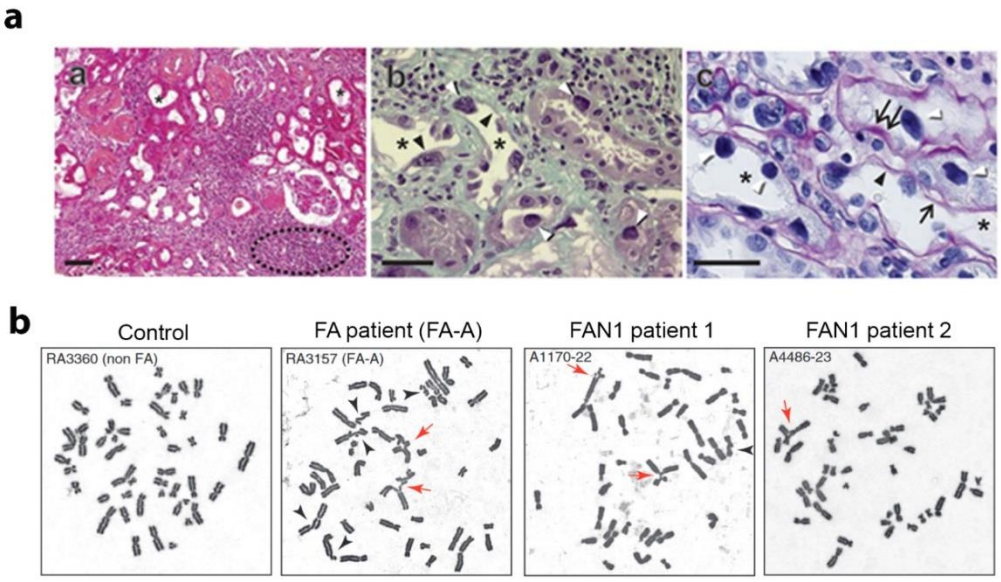

Fig. 8 - Mutations in *FAN1* induce KIN. Modified from Zhou *et al*. (2012).

a) Histopathology from a patient carrying mutations in the *FAN1* gene. Widespread fibrosis in blue-grey stained by Masson's Trichrome in the left panel. Karyomegalic cells (white arrow heads) and tubular dilation (asterisks) in the central and right panel.

b) Presence of radial chromosomes (red arrows) and chromosome breaks (black arrows) upon MMC treatment in metaphase spreads of cells isolated from non-FA, FA patients and two individuals carrying mutations in *FAN1* gene.

Other clinical cases of KIN in humans have been reported without a clear association to a specific genetic defect. In many patients, the development of KIN has been explained through the contact with the mycotoxin Ochratoxin A (OTA), after the detection of this compound in blood and urine (Hassen *et al*., 2004, Godin *et al*., 1996). Consistently, OTA nephrotoxicity and renal carcinogenesis has been proven in mice and rats (Adler *et al*., 2009, Vettorazzi *et al*., 2011, Taniai *et al*., 2014). While the actual mechanism of action of OTA is not well understood, however, there is evidence that OTA disrupts renal tubuli by generating DNA damage (Cosimi *et al*., 2009, Mally *et al*., 2005, Mosesso *et al*., 2008, Mally, 2012). In addition, OTA has been shown to behave as a catalytic inhibitor of Topoisomerase II, providing a potential link of the disease to topological stress (Cosimi *et al*., 2009). To further support the link with DNA damage, KIN has been also linked to treatment with cytotoxic drugs used in the clinic as chemotherapeutics. For instance, features of KIN have been reported in cancer patients treated with busulfan and ifosfamide (McCulloch *et al*., 2011, Mihatsch *et al*., 1979, Burry, 1974, Godin *et al*., 1996, Jayasurya *et al*., 2016).

3 - Structural maintenance of Chromosomes

The accurate transmission of the genetic information from mother to daughter cells is of crucial importance for the survival of an organism. Therefore, the maintenance of genomic integrity is highly controlled. One of the pathways that ensures the correct chromosomal architecture and organization involves the Structural Maintenance of Chromosomes (SMC) family.

Each SMC protein consists of 1000-1500 aminoacids and contains globular N- and C-terminal ATPase domains (Walker A and B motifs) linked by two long α-helixes folded against each other at a central globular hinge domain (Jacome *et al.*, 2015, Jeppsson *et al*., 2014, Murray and Carr, 2008, Losada and Hirano, 2005) **(Figure 9a)**. While a single SMC protein is present in bacteria, six have been identified in eukaryotes. All eukaryotic SMC complexes are organized into SMC protein heterodimers, all linked through the hinge region, whereas the non-SMC subunits hold the ATPase domains together (Murray and Carr, 2008, Verver *et al*., 2016). In eukaryotes, these six SMC proteins are organized into three independent heterodimeric complexes named SMC1/3 (cohesin), SMC2/4 (condensin), and SMC5/6 complex **(Figure 9b)**.

The cohesin complex is essential to maintain the cohesion of sister chromatids. Cohesins generate a counterforce to the mitotic spindle and facilitate chromosome alignment. Condensins, on the other hand, promote the compaction and the disentanglement of sister chromatids. While the role of cohesins and condensins in regulating chromosome architecture is well characterized, the true function of the SMC5/6 complex function is still not fully understood (Jeppsson *et al*., 2014).

Although prokaryotic SMC proteins share higher sequence and structural similarity with cohesins and condensins, SMC5/6 appears more closely related to the prokaryotic complex in terms of phenotype similarity when these proteins are mutated. This observation suggests that the SMC5/6 complex have diverged faster than cohesins and condensins from their last common ancestor. It has been recently proposed that the SMC complexes derive from a SMC5/6-like common ancestor, from which a cohesin-condensin-like common ancestor originated, and later cohesins and condensins evolved through a series of gene duplication events (Palecek and Gruber, 2015).

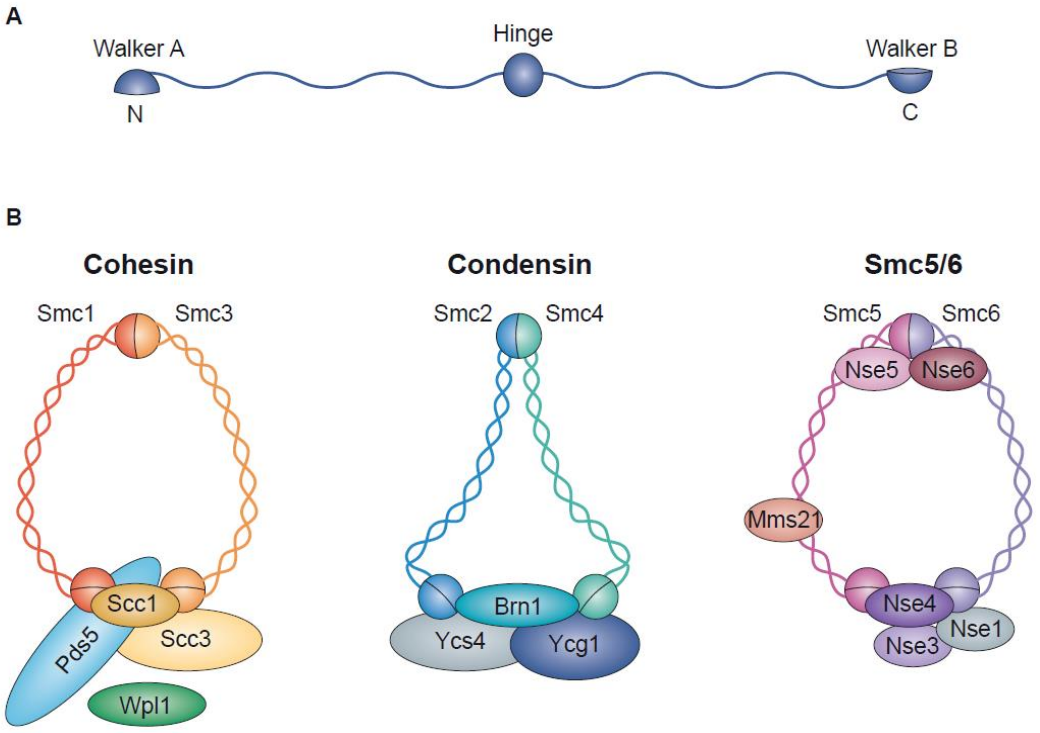

Fig. 9 - Architecture of SMC complexes in yeasts (Jeppsson *et al.*, 2014).

a) Structure of unfolded SMC proteins. Each SMC protein has a central globular hinge domain. The hinge domain is flanked by coiled-coil regions, both of which end with a globular domain (Walker A or a Walker B motifs). The coiled-coil domains associate through antiparallel interaction, bringing the Walker A and B motifs together to form the ATPase domain of the SMC protein.

b) Members of SMC family (cohesins, condensins and SMC5/6 complex) in eukaryotes and composition. In each complex, two SMC proteins interact through the hinge domain and assume a V-shape structure. These subunits are Smc1 and Smc3 for the cohesin complex, Smc2 and Smc4 for the condensin and Smc5 and Smc6 form the SMC-5/6 complex. Complex-specific non-SMC subunits further associate to the SMC heterodimers. Kleisin proteins held together the globular head domains of the SMC proteins and allow the heterodimer to adopt a ring-like structures. Non-SMC members of the cohesin complex are Pds5, Wpl1 and the kleisin subunit Scc1 and Scc3. In yeast, Brn1 bridges the Smc2 and Smc4 head domains and Ycs4 and Ycg1 further associate to the complex. Beside the SMC heterodimer in the SMC-5/6 complex, 6 additional elements are part of the complex: Nse1, Nse2 (Mms21), Nse3, the kleisin subunit Nse4, Nse5 and Nse6.

3.1 - Architecture of the SMC5/6 complex

The SMC5/6 complex is composed of two SMC elements, SMC5 and SMC6 (the SMC5/6 heterodimer), and 6 non-SMC elements named Nse1-6 in yeasts (NSMCE1-6 in higher organisms). A second level of organization has been described in yeasts where there is evidence of the subunits being organized into three subcomplexes. One of these subcomplexes is formed by the two SMC proteins and Nse2 bound to the coiled-coil domain of Smc5 (Duan *et al.*, 2009). Interestingly, a Smc5-Nse2 dimer lacking of Smc6 might exist in

mitosis outside the complex (Stephan *et al*., 2011). Nse1, Nse3 and Nse4 associate together to the globular heads of the SMC proteins. Nse5 and Nse6 form a dimer that in budding yeasts interacts with the hinges domains of Smc5 and Smc6 and in fission yeast associates with the globular heads (Stephan *et al*., 2011, Kegel and Sjögren, 2010).

Beside the ATPase activity of the SMC proteins, small ubiquitin modifier (SUMO) ligase and ubiquitin ligase activities have been described. Nse1 and Nse2 contain RING fingers domains, suggesting the ability of these proteins to conjugate ubiquitin or ubiquin-like proteins to protein targets. Nse1 carries a RING finger domain enabling ubiquitin modification. Nse2 contains a catalytic SP-like ring and belongs to Siz1/PIAS (SP) family of E3 SUMO-ligases (Stephan *et al*., 2011). Sumoylation induced by Nse2 does not result in mono- o di-sumoylation but poly-sumoylation and/or multiple sumoylation attaching sites. Nse2 targets include components of the SMC5/6 complex, Nse2 itself, shelterin subunits and Ku70 (Kliszczak *et al*., 2012). Although, most of SUMO ligases contain a SAP sequence required for DNA binding, Nse2 does not have this motif and bind DNA through its interaction with the SMC5/6 complex (Duan *et al*., 2009).

3.2 - The enigmatic role of the SMC5/6 complex in chromosome maintenance

Since, besides the ATPase activity of the globular domains of SMC5 and SMC6, the ubiquitin ligase Nse1 and the SUMO-ligase Nse2 are the only known enzymatic activities within the complex, it was assumed that the mechanisms to preserve chromosomes structure by SMC5/6 complex occurred exclusively through their activities (Fernandez-Capetillo, 2016). However, the abrogation of Nse1 or Nse2 enzymatic activities in mutant yeasts does not affect viability, whereas the deletion of these proteins is lethal (Andrews *et al*., 2005, Pebernard *et al*., 2008a). These findings suggest that the role of these enzymatic activities could be rather a fine tuning one, whereas the role of the actual SMC5/6 in structural integrity would be what makes it essential. Accordingly, all the components of the complex are required for viability in *Saccharomyces cerevisiae* and all except Nse5 and Nse6 are essential in *Schizosaccaromyces pombe* (Stephan *et al*., 2011).

The SMC5/6 complex shows a dynamic pattern of localization throughout the cell cycle. For instance the complex binds origins of replication in S-phase, while associate to centromeres and other chromosomal regions in G2-M (Lindroos *et al*., 2006, Menolfi *et al*., 2015, Bustard *et al*., 2012). In a recent study in yeast, SMC5/6 functions were restricted to either S-phase or G2-M, by using cell cycle-regulated alleles in order to separate the potential functions of the complex and to evaluate the outcome of their loss (Bustard *et al*., 2012, Menolfi *et al*., 2015). While restricting the function of SMC5/6 to G2-M had no effect on viability, its absence

in G2-M was lethal. Therefore, the essential functions of the SMC5/6 complex are due to its roles in G2-M (Menolfi *et al*., 2015). Moreover, expression of the complex in mitosis is sufficient to enable the growth of SMC5/6 complex deficient yeast strains, suggesting that its role is similar to the one of the BLM helicase and linked to the dissolution of HJs (Bermúdez-López *et al*., 2010).

The first evidence of the SMC5/6 complex being involved in genome maintenance comes from the identification of rad18 (Smc6) and mms21 (Nse2) mutants as being sensitive to the alkylating agent MMS (Prakash and Prakash, 1977, Nasim and Smith, 1975). Mutations inactivating Nse2 SUMO ligase activity in yeast also sensitize cells to a variety of genotoxic agents, including hydroxyurea (HU), IR, UV and MMS, although to a much lower extent to the sensitivity observed in null alleles (Andrews *et al*., 2005, McDonald *et al*., 2003, Stephan *et al.*, 2011).

Epistatic relationships between the SMC5/6 complex and HR repair factors supports the implication of the complex in HR repair. For instance, IR sensitivity of Smc6 mutants in yeast could be rescued by deletion of RAD51, one of the main actors of HR (Stephan *et al*., 2011, Lehmann *et al*., 1995). Interestingly, the absence of SMC components does not affect early events of HR repair, such as RAD51 recruitment (Stephan *et al*., 2011). These observations place the SMC5/6 complex in the latest steps of HR after RAD51-mediated strand invasion and D-loop formation. On the other hand, it has been argued that the function of the complex may go beyond HR and DNA repair, since every member of the complex is essential for life in yeast, whereas HR is not. Moreover, not only HR can still occur in absence of functional SMC5/6, in fact the outcome of repair is dramatically affected with increased SCE levels and recombination (Stephan *et al*., 2011). As such, Nse2 mutant yeasts, but also chicken SMC5 knockout cells and hypomorphic SMC6 mice, are all characterized by increased rate of recombination (Prakash and Prakash, 1977, Kliszczak *et al*., 2012, Ju *et al*., 2013).

Whereas the role of SMC5/6 in HR is unclear, a widely accepted role for the SMC5/6 complex consists in the processing of sister-chromatids linkages, as exemplified by the accumulation of cruciform structures in mutant yeasts (Ampatzidou *et al*., 2006, Branzei *et al*., 2006, Bermúdez-López *et al*., 2010, Yong-Gonzales *et al*., 2012, Sollier *et al*., 2009). Some of these structures represent the products of HR repair that remain entangled. In support of this, RAD51 deletion prevents the accumulation of these linked molecules in SMC5/6 mutants and alleviate overall sickness of the mutants (Fernandez-Capetillo, 2016, Torres-Rosell *et al*., 2005, Yong-Gonzales *et al*., 2012). This phenomenon is remarkably similar to that observed in BLM-deficient cells, where joint DNA molecule dissolution is impaired. Hence, in the absence of a functional SMC5/6 complex, the dissolution pathway

that is in charge of processing these repair intermediates without cross-over appears abrogated and joint DNA molecules are instead repaired by the recombination-prone pathway of resolution, explaining the observed increase in SCE events.

In regards to the nature of these joint molecules, the SMC5/6 complex operates preferentially at repetitive sequences that are difficult to replicate and prone to fork stalling and collapse. Indeed, Smc5/6 localizes at natural replication pausing sites, centromeres and telomeres (Ampatzidou et al., 2006, Lindroos et al., 2006, Menolfi et al., 2015, Pebernard et al., 2008a, Pebernard et al., 2008b). Replication problems occurring at these loci are in fact repaired by HR through the generation of recombination intermediates. Hence, in the absence of Smc6 joint DNA molecules accumulate at ribosomal arrays (rDNA repeats) regions, the biggest repeat in yeast, and murine NSMCE2 accumulates at pericentromeric DNA, the biggest mammalian repeat (Torres-Rosell *et al*., 2005, Lindroos *et al*., 2006, Fernandez-Capetillo, 2016). Besides its involvement in joint DNA molecule dissolution, or perhaps linked to it, several works have shown that the role of SMC5/6 is linked to chromosomal topology, and synthetic lethal interactions of Smc5/6 mutants with Topoisomerase mutants have been reported (Takahashi *et al*., 2008, Outwin *et al*., 2009). Related to this role, the analysis of SMC6 localization revealed a distinct role of the complex in the maintenance of long chromosomes (Kegel *et al*., 2011, Lindroos *et al*., 2006), which was also related to Topoisomerase activity.

Altogether, although the initial role of SMC5/6 was placed in HR, it is becoming more and more evident that the complex holds essential HR-independent functions that are related to chromosome topology and segregation, which are yet to be fully understood.

3.3 - Mutations of SMC5/6 complex in humans

So far, two genetic disorders have been described to arise from mutations in components of the SMC5/6 complex (Payne *et al*., 2014, van der Crabben *et al*., 2016). Since complete depletion of any components is lethal, these mutations do not completely abrogate the expression of the protein and preserve some functions of the SMC5/6 complex that are compatible with life.

First, two female patients displaying a variety of pathological features, including severe primordial dwarfism, facial dysmorphism, extremely insulin resistant diabetes, fatty liver, hypertriglyceridemia and primary gonadal failure, were found to carry distinct heterozygous frame-shift mutations in the SUMO ligase *NSMCE2*. One of these mutations occurs between the N-terminal and the SP-RING domain and introduces 18 unstructured amino acids. The

other results in the removal of 14 C-terminal amino acids of the protein. In both cases, the mutations destabilize the protein levels of NSMCE2, resulting in lower protein expression. Due to certain similarities, one of the patients was initially examined for potential diagnosis of Fanconi Anaemia or Bloom syndrome, but the diagnostic tests for such conditions were negative. However, the karyotype analysis showed a moderate number of chromosomal breaks. Furthermore, cells isolated from the patients presented increased frequency of micronuclei and bridges formation after hydroxyurea (HU)-induced replication fork stalling (Payne *et al*., 2014). Due to the low number of patients and the absence of long-term evaluation, the full outcome of this disease is yet to be known.

Besides *NSMCE2* mutations, two missense mutations in *NSMCE3* were also found in 4 patients. These mutations lead to a very different clinical outcome characterized by severe pulmonary disease, immune deficiency and increased infection susceptibility. *In vitro* investigations indicate that one of the mutations leads to a C-terminal truncated NSMCE3, whereas the other variant shows a reduced interaction with NSMCE4 and SMC6. The *NSMCE3* mutations disrupt its interaction within the SMC5/6 complex leading to a more severe destabilization of the complex compared to the one observed in cells isolated from individuals carrying *NSMCE2* mutations. In this case, lymphocytes of affected individuals showed chromosome rearrangements, breakages and micronuclei and their fibroblasts showed sensitivity to different DNA-damaging agents (van der Crabben et *al*., 2016). The increased severity of the mutation is likely responsible for the discrepant phenotypes that are observed in both diseases.

In summary, while initially associated to HR, the true function of the SMC5/6 complex remains to be truly understood. The analysis of the phenotypes in mouse models and human patients often helps to get a deeper understanding of the physiological role of a given protein or complex. Here, I report my research using mouse models of NSMCE2, which led us to discover a systemic role of SMC5/6 in chromosome segregation, but also a distinct role for NSMCE2, independent of its SUMO ligase activity, in kidney function; and which supports that the main role of SMC5/6 is linked to Topoisomerase activity.

OBJECTIVES

Objectives

- To analyze the recruitment of NSMCE2 to sites of DNA damage and inter-strand crosslinks.
- To determine the role of NSMCE2 in DNA replication and chromosome segregation.
- To investigate the consequences of *Nsmce2* deletion in adult mice.
- To characterize the impact of NSMCE2 deficiency specifically in the kidneys.
- To explore the role of NSMCE2 in the cellular response to topological stress.
- To determine the functional redundancy of NSMCE2 and FAN1 in response to topoisomerase inhibition.

MATERIALS AND METHODS

Materials and Methods

1 - Mouse work

1.1 - Maintenance of mice lines and genotyping

Mice used for this study were kept under standard conditions at the pathogen-free facility of the Spanish National Cancer Research (CNIO). All mouse work was performed in accordance with the Guidelines for Humane Endpoints for Animals Used in Biomedical Research, and under the supervision of the Ethics Committee for Animal Research of the "Instituto de Salud Carlos III".

In order to genotype the animals, DNA was isolated from small pieces of the animal tail and digested for 16 hours at 55°C with 100 mM NaCl, 50 mM Tris-HCl pH 8, 5 mM EDTA, 1% sodium dodecyl sulfate solution (SDS, Sigma) and 400 µg/ml proteinase K (Roche). After treating the cellular lysate with saturated 6M NaCl solution, DNA was precipitated with isopropanol, washed with 70% ethanol and resuspended in distilled water.

GoTaq® DNA Polymerase (Promega) was used for genotyping and PCR was carried out according to manufacturer's instruction. Genotyping PCRs were performed in a reaction mix composed of 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1X green GoTaq® reaction buffer, 1.25 U GoTaq® DNA polymerase, 0.5 µM of each oligonucleotide, 100 ng of genomic DNA and nuclease free water up to 20 µl.

The following primers were used to genotype the *Nsmce2*^{lox} allele:

Forward: ATTAGAAAATAATGATCCTAGGAAACATTAATTCTGCAACTCGTTGTT
Reverse: TAAATACTCATGCCTTTCTTACCCCGTCTTTCT
A band of 150 bp for the wild-type *Nsmce2* allele and a band of 250 bp for the *Nsmce2*^{lox} allele are expected.

The following primers were used to genotype the *Nsmce2*^{KI} allele:

Forward: GGTGGGACTGGAGTATTGGA
Reverse 1: TATGTGCGTTGAGTGTGCAA
Reverse 2: CAGAAAGCGAAGGAGCAAAG

A band of 480 bp for the wild-type *Nsmce2* allele and a band of 560 bp for the *Nsmce2*^{KI} allele are expected.

In order to genotype the presence of the UQ.Cre^{ERT2} recombinase the following primers were used:

Forward: CGATGCAACGAGTGATGAGGTTC
Reverse: GCACGTTCACCGGCATCAAC
A band of 350 bp is expected when the UQ.Cre^{ERT2} recombinase allele is present.

In order to genotype the CD19-Cre recombinase allele the following primers were used:

Forward: AGGGAGGCAATGTTGTGCT
Reverse 1: GTTCGAACGCTAGAGCCTGTTT
Reverse 2: TGCCAGACCAAAGAACTTCCTC
A band of 600 bp for the wild-type allele and a band of 350 bp for the CD19-Cre^{KI} allele are expected.

For *Nsmce2* deletion in UQ.Cre^{ERT2} mice, animals were fed with a diet containing 400 mg of 4-Hydroxytamoxifen (4-OHT) per kg (Harlan Tekland, Madison, USA), starting at weaning.

1.2 - Blood test

Blood was collected from mice either from the orbital sinus (peri-orbital bleeding) with anaesthetized animal or by cardiac puncture after the animals were sacrificed. Blood serum was obtained by centrifugation and the levels of renal markers were evaluated by using the ABX Pentra 400 apparatus. To this end, quantitative *in vitro* determination of urea (Urea CP ABX Pentra – Horiba), creatinine (ABX Pentra Enzymatic Creatinine CP-Horiba) and albumin (ABX Pentra Albumin CP – Horiba) was carried out.

1.3 - Immunohistochemistry

Tissue samples were embedded in formalin and processed at CNIO's Pathology Unit. 2.5 µm tissue sections were treated with citrate for antigen recovery and processed for p-Ser139 H2Ax (05-636 Millipore) staining following standard procedures. To evaluate kidney

architecture tissue sections were stained with Hematoxilin and Eosin. Levels of fibrosis were evaluated by Masson's Trichrome staining. Kidneys sections were digitalised by using the MIRAX system (Zeiss) and the images were captured through the ZEN 2 lite software (Zeiss). In order to analyse the renal nuclear area, several images for each renal compartments were captured and analysed by the imageJ software.

2 - Cellular biology

2.1 - Cell culture

Unless otherwise specified, all cell lines were cultured in DMEM media (4.5 g/L Glucose; L-Glutamine; Lonza, Switzerland) with 10%-15% of inactivated fetal bovine serum (FBS) (South American Origin, Lonza) and a mix of penicillin and streptomycin (Gibco, Invitrogen, Life Technologies, Carlsbad, CA). Cells were kept in incubators at 37°C and 5% $C0_2$, except for mouse embryonic fibroblasts (MEFs), that were maintained in hypoxia incubators at 37°C, 5% $C0_2$ and 5% O_2.

Deletion of *Nmsce2* in UQ. Cre^{ERT2} $Nsmce2^{lox/lox}$ was induced by adding 1 µM 4-Hydroxytamoxifen (4-OHT, Sigma) to the culture media for at least 48h before the corresponding assay.

Methyl methanesulfonate (MMS, Sigma), Mitomycin C (MMC, Sigma), ICRF-193 (Sigma) and Etoposide (Sigma) were used to induce distinct types of DNA damage. 10 Gy IR treatment was carried out to induce damage and cells were left to recover for one hour before proceeding to the corresponding assay.

2.2 - Isolation of MEFs

Mice were selected in order to obtain pups with the genotype of interest. At 13.5 days of gestation, the female was sacrificed and the embryos were isolated from the mother in sterile conditions. The fetal liver was then removed and a piece of head was also isolated for genotyping analysis. The rest of the embryo was chopped with a sterile blade and incubated for 10 minutes with 1 ml trypsin-0,25% EDTA (Gibco). In order to dissolve the aggregates, the solution was mixed vigorously and the trypsin was neutralized by adding 9 ml DMEM media (Gibco) supplemented with 15% inactivated fetal bovine serum (FBS) and 1%

penicillin/streptomycin (Gibco, Invitrogen, Life Technologies, Carlsbad, CA). Isolated MEFs were then transferred to a 100 mm^2 petri dish and kept in hypoxia incubators (5% O2).

2.3 - Isolation of splenic B-lymphocytes

In order to isolate B-lymphocytes from mice spleen, a spleenectomy was carried out. Spleens were squeezed onto a 40 µm filter with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) with 1% bovine serum albumin (BSA Fraction V, Roche) and collected into a 15 ml falcon tube. Red blood cells were lysed with a hypotonic buffer solution (ACK Lysing Buffer, Lonza). After 5 minutes incubation, the buffer was inactivated by adding 10 ml of RPMI media (Euroclone). Cells were washed twice with 1X PBS/1% BSA and then passed through a 40 µm filter to remove aggregates. Cells were centrifuged again and resuspended in 920 µl 1X PBS/1%BSA and 80 µl of anti-CD43 antibody conjugated magnetic beads (Miltenyi Biotech, Germany). After 15 minutes incubation at 4°C, cells were washed again and resuspended in 1 ml PBS/1%BSA. The solution was transferred into a separating column (MS column, Miltenyi) previously equilibrated with 1 ml PBS/1%BSA and standing in a magnetic scaffold (OctoMACS separator, Miltenyi). B-lymphocytes were not retained by the magnetic field of the column and were collected into a tube.

$1x10^6$ primary B-lymphocytes per ml were finally seeded with RPMI supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco, Invitrogen), 2 mM glutamine (Gibco, Invitrogen), non-essential aminoacids (Lonza), sodium pyruvate (Gibco, Invitrogen), 50 mM b-mercaptoetanol (Gibco, Invitrogen) and 10 mM HEPES (Lonza) and 25 µg/ml LPS (Sigma-Aldrich, St. Louis, MO) was added to induce proliferation.

2.4 - Transient expression of proteins

Cells were seeded in P24-well plates in order to reach 90-95% confluence at the time of transfection. In order to evaluate the potential colocalization between FAN1 and NSMCE2, GFP-FAN1 was overexpressed transiently in 3T3 cells. To this end, 3T3 cells were transfected with the plasmid pcDNA5 FRT/TO.GFP.Puro.DU FAN1 kindly provided by Dr. John Rouse. For each well, 0.5 µg of the plasmid was mixed with 100 µl Optimem® Medium (Life Technologies). After a 5 minutes incubation, the mix was combined with 100 µl Optimem® Medium and 1 µl Lipofectamine™ 2000 (Life Technologies) previously mixed. The solution was further incubated for 20 minutes to allow the DNA-Lipofectamine™ 2000 complexes to form. The solution was finally added to the cells by gentle mixing.

2.5 - Production of viral particles and stable expression of transgenes

In order to produce virus particles, 293T cells were transfected with the appropriate viral packaging plasmid and with the plasmid containing the desired protein. The media was changed 12 h after the transfection and the virus was collected 24 hours later. Before using the viral supernatant for the infection, the media was filtered through a 0.45 µm filter. The virus was added to cells seeded in 6-well plates and spun at 1850xg for one hour. Then, the media was changed and antibiotic selection was carried out after 48h from the infection. MEFs were immortalised by the expression of the T121 antigen that was stably integrated by using a retroviral system. To this end, pBabe-puro-T121 and pCL-Eco, kindly provided by Dr. Manuel Serrano, were transfected in 293T to produce virus particles as previously described.

2.6 - Generation *Fan1* knockout cells with CRISPR-Cas9 technology

Fan1 knockout MEFs were generated by using the CRISPR-Cas9 technology. Three different sgRNAs targeting the first exon of the murine *Fan1* allele were cloned into the lentiviral vector LentiCRISPv2 (Addgene #52961). The following oligos were used:

sgRNA-*Fan1*-1

CACCGACTCATCAAGGTGCCGAATG
AAACCATTCGGCACCTTGATGAGTC

sgRNA-*Fan1*-2

CACCGCGTTCAAGTAGAGCCTGCTC
AAACGAGCAGGCTCTACTTGAACGC

sgRNA-*Fan1*-3

CACCGCTGGTAGATAAGCTTCTACG
AAACCGTAGAAGCTTATCTACCAGC

Individual lentiviral vectors were co-transfected with 3rd generation packaging vectors (pMDL, Rev and VSVg) in 293T cells using Lipofectamine 2000 (Invitrogen) in order to generate viral supernatants as described by Ruiz *et al.* (2011). MEFs were finally infected by using a mixture of the three viral supernatants.

2.7 - Immunofluorescence and high-throughput microscopy (HTM)

For high-content screening cells were cultured in µCLEAR bottom 96-well plates (Greiner Bio-One) and treated according to the experimental protocol. Cells were fixed with 4% paraformaldehyde (EMS)/PBS at room temperature for 10 minutes and permeabilized with 0.1% sodium citrate/0.1%Triton X-100 (Sigma)/PBS for 15 minutes. After washing three times with 0.25% BSA/0.1% Tween20 (Sigma)/PBS, cells were incubated in blocking solution (2.5% BSA, 0.1% Tween20/PBS) for 30 minutes. The corresponding primary antibody diluted in blocking solution was incubated over-night at 4°C. After washing 3 times, the secondary antibody conjugated to fluorophore was added for 1 hour at room temperature and nuclei were stained with DAPI.

Staining with certain antibodies required a pre-extraction step to prevent the cytoplasm to mask nuclear signal. Indeed, cells were treated 3-5 minutes with CSKI pre-extraction solution (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA and 0.5% Triton X-100) and, after washing 5 times with 0.25% BSA, 0.1% Tween20 in PBS, cells were fixed with mSTF buffer (150 mM 2-bromo-2-nitro-1,3,-propanediol, 108 mM diazolidinyl urea, 10 mM Na citrate, 50 mM EDTA pH 5.7) for 30 minutes. The preparation was then permeabilised with 100 mM Tris-HCl pH 7.4, 50 mM EDTA pH 8 and 0.5% Triton X-100. Blocking and immunostaining were performed as previously described.

Images were automatically acquired by an Opera High-Content Screening System (Perkin Elmer). A 40 x magnification lens was used and pictures were taken at non-saturating conditions. Images were segmented using the DAPI staining to generate masks matching cell nuclei. The number of foci and the intensity average signal of the desired protein within the nuclei area were calculated. Data were represented with the use of the Prism software (GraphPad Software).

To evaluate protein colocalization, cells were seeded on a glass coverslip treated with gelatin, in a P24 well plate. Cell preparation and immunostaining was performed as described. z-stack images were acquired by using a confocal microscope and colocalization was evaluated at each layer.

2.8 - Laser irradiation induced damage

Analysis of proteins recruitment to laser-induced damage was carried out at the microscopy facility of the MRC Protein Phosphorylation Unit in Dundee during a short visit for training purposes in the lab of Dr. John Rouse.

Cells seeded in 35-mm glass-bottomed dishes were incubated for one hour with 50 µM trimethyl-psoralen (TMP Sigma-Aldrich) or 50 µM angelicin (Sigma) to induce respectively laser stripes of inter- and intra-strand crosslink after UV-A irradiation.

Before proceeding to the UV-A irradiation step, the culture media was replaced with a colourless media (Leibovitz's, Gibco), to minimize laser diffraction. A 355-nm UV-A laser attached to a PALM microscope (Zeiss) was used to irradiate a track along cell nuclei as previously described (Lachaud *et al*., 2014). The power of the laser (percentage of intensity) was set to 20% to generate ICLs, and the areas were struck at low speed. 30 minutes after the UV-A irradiation, cells were then pre-extracted with 0.5% Triton X-100 in PBS and fixed with 4% paraformaldehyde. Immunostaining was performed as described and γH2Ax was used as a positive control of recruitment.

2.9 - Chromosomes spread preparation

Colcemide was added at 0.1 µg/ml during the last 2-5 hours of cell culture. Cells were then collected, washed and resuspended in hypotonic buffer (0.075 M potassium chloride) at 37°C. After 15 minutes incubation, cells were centrifuged and the cell pellet was gently resuspended in methanol: glacial acetic acid (3:1) fixative solution and incubated for 20 minutes. Cells were then washed with fixative and a few drops were released onto an alcohol cleaned slide. Slides were finally stained with Giemsa solution and let the slide dry.

For SCE analyses, cells were cultured in the presence of BrdU (Sigma) at a final concentration of 10 µM and then allowed to replicate their DNA once for 24 hours. Metaphases were prepared using the standard procedure described above. Metaphase spreads were subsequently treated with UV (355 nm) and Hoechst to counterstain nascent DNA strands.

2.10 - Chromosome Orientation Fluorescence In Situ Hybridization (CO-FISH)

CO-FISH was performed according to the procedure published by Gonzalo *et al.* (2006). Cells were cultured for 16 hours in the presence of 5'-bromo-2'-deoxyuridine (BrdU; Sigma, St Louis, MO) at a final concentration of 10 µM. Colcemid was then added at a concentration of 0.1 µg/ml for the last hour of incubation. Cells were recovered and metaphase spreads were prepared as previously described.

In order to degrade the newly synthetized DNA strand the slides were treated with 0.5 mg/ml RNase A, followed by staining with 0.5 µg/ml Hoechst 33258 (Sigma) in 2x SSC. DNA was

then exposed to UV light (365 nm), digested first with 3U/µl exonuclease (Promega) and later with 1mg/ml pepsin (Sigma).

A first probe (TTAGGG) labelled with Cy3 and a second probe (CCCTAA) labelled with Rhodamine green (Applied Biosystems, Bedford, MA) were used. Metaphase spreads were captured on a Leitz Leica DMRB fluorescence microscope.

2.11 - DNA fiber assay

Cells were pulse-labelled with 50 µM CldU for 20 minutes, followed by 250 µM IdU for other 20 minutes. Labelled cells were collected, and DNA fibers were spread in buffer containing 0.5% SDS, 200 mM Tris pH 7.4, and 50 mM EDTA. DNA was fixed with freshly made 3:1 methanol:acetic acid for 2 minutes and slides were treated with 2.5 M HCl for 30 minutes.

To visualize labelled tracks, fibers were blocked with 1% BSA, 0.1% Triton X-100 in PBS and incubated with primary antibodies (for CldU, rat anti-BrdU; for IdU, mouse anti-BrdU) Mouse anti-ssDNA antibody was used to assess the integrity of the fibers. Slides were finally developed with Alexa-conjugated secondary antibodies. Slides were examined with a Leica DM6000 B microscope, as described previously (Mourón *et al*., 2013). The conversion factor used was 1 µm = 2.59 kb (Jackson and Pombo, 1998). In each assay, 200–300 tracks were measured to estimate fork rate and 300–500 tracks were analyzed to estimate the frequency of origin firing.

2.12 - Flow cytometry analysis

2.12.1 - Cell cycle analysis

Cell cycle profiles were analysed by staining B-cells and MEFs with Hoechst staining or propidium iodide (PI) staining. For Hoechst staining, one million cells were washed and resuspended in pre-warmed culture medium supplemented with 1 µg/ml Hoechst 33342 (Sigma). The mix was then incubated at 37°C for 30 minutes.

The samples were otherwise fixed with 70% ethanol over-night and resuspended in PBS containing 10 µg/ml propidium iodide (PI, Sigma) and 0.5 mg/ml RNaseA.

Analytic flow profiles of DNA content were recorded on BD FACSCanto II cytometer. A minimum of 10,000 events were counted. Data was analyzed with Flowjo software (XY).

2.12.2 - Viability assay by DAPI exclusion

To assess the sensitivity to ICRF-193, B-cells were stained with DAPI, exploiting the ability of the dye to stain exclusively dead cells. Cells were washed and resuspended in 0.2 mg/ml DAPI in PBS. Fluorescence data was acquired by BD FACSCanto II cytometer and data was analysed by Flowjo software (XY).

2.13 - Clonogenic assays

The ability to form colonies was assessed in immortalised *Nsmce2* conditional knockout MEFs. 1000 cells were plated in P6-well plates without and in presence of 4-OHT and the media was replaced every 2 days. Cells were kept in culture for 10 days and colonies were stained with 0.33% methylene blue (Sigma) in methanol.

2.14 - CellTiter-Glo® Luminescent Cell Viability Assay

Defects in cell proliferation and drugs sensitivity were assessed by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). *Nsmce2* conditional knockout MEFs were seeded in µCLEAR bottom 96-well plates (Greiner Bio-One) and *Nsmce2* deletion was induced by treatment with 4-OHT for 48 hours. Cells were then exposed to a range of ICRF-193 concentration for 48 hours. Chemoluminescence was monitored by the Victor Multiwell reader plate (PerkinElmer) 20 minutes after the incubation with the CellTiter-Glo® reagent.

3 - Molecular biology and biochemistry

3.1 - Antibodies

The following primary antibodies were used: TOPOII (Topogen TG2011-1), NSMCE2 generated in house, FAN1 (kindly provided by Dr. John Rouse), a-Tubulin (Sigma T-9026), H2A (Cell Signaling 3636), γH2AX (Upstate Biotechnology, 05-636), SMC5 (Biothyl, A300-236A) and SMC5 (kindly provided by Prof. Alan Lehman), H3S10P (Millipore 05-806) and H3K93m (Millipore 07-442).

3.2 - In house antibody production

Due to the lack of reagents to study NSMCE2 in mammals, we have generated in house antibodies against the murine NSMCE2 protein.

The cDNA of murine *Nsmce2* was cloned into pET-45b (Novagen 71327), which allows the expression of the desired protein fused to N-terminal 6xHis-tag. NSMCE2 protein was expressed in *Escherichia coli* BL21 and purified by Ni^{+2} affinity chromatography. The purified protein was then dialysed by using Slide A-lyser Dialysis Cassettes (Pierce).

The recombinant full-length murine NSMCE2 fused to His-tag was used to immunize two rabbits in order to generate rabbit polyclonal (VivoTecnia, Spain). The antibodies were the purified using Affi-Gel columns (Bio-Rad) previously coated with immobilized GST-NSMCE2 protein.

3.3 - Cellular fractionation and immunoblotting

For whole cell protein extracts cells were washed and resuspended in 50 mM Tris pH 7.09 / 8M Urea / 1% Chaps and placed at 4°C on agitation for at least 30 minutes.

Cytosolic and nuclear extracts were prepared as previously described (Lecona *et al*., 2008). In order to isolate the cytosolic fraction cells were resuspended with ice-cold hypotonic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA containing protease and phosphatase inhibitors) and incubated for 10 minutes on ice. Then, NP-40 (Sigma) was added and after 3 minutes, the samples were mixed, centrifuged at 2500g for 2 minutes and the supernatant was collected (cytosolic fraction). The pellet was then resuspended in high-salt concentration extraction buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA containing protease and phosphatase inhibitors) and the extract was left shaking at 4°C for at least 30 minutes. The samples was spun down at maximum speed and the supernatant collected (soluble nuclear fraction). In order to obtain chromatin bound-proteins, 50mM Tris pH 7.9/ 8 M Urea/ 1% Chaps was added to the pellet that was incubated with shaking at 4˚C for at least 30 minutes. The extract was centrifuged at maximum speed and the supernatant collected (chromatin fraction). In order to isolate the total nuclear extract, high-salt-concentration extraction buffer (20 mM HEPES, pH 7.9, 0.6 M NaCl, 1 mM EDTA containing protease and phosphatase inhibitors) was added to the pellet after the isolation of the cytosolic fraction. The sample was incubated shaking at 4°C for 10min, sonicated briefly until it was clear and incubated for at least 30 minutes more. The total nuclear extract was then isolated by centrifugation at max speed. Protein concentration of the extracts was measured by Bradford method.

NuPage loading buffer (Life technologies) was added to the normalised samples that were heated for 10 minutes at 70°C, and separated in gradient gels 4-12% SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel, Invitrogen). Proteins were subsequently wet-transferred to nitrocellulose membranes (Hybond ECL Nitrocellulose, Amersham). After transfer, membranes were blocked for 30 minutes at room temperature in 5% skimmed milk (Central Lechera Asturiana) in TBS/0.1% Tween20 (TBS-T) and incubated overnight at 4°C with primary antibodies diluted in TBS-T/5% BSA. After washing the membranes 3 times with TBS-T, they were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies.

Proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Life technologies) on a ChemiDoc XRS Gel Photo Documentation System (Bio-Rad).

3.4 - Co-immunoprecipitation

Proteins interaction was evaluated in total nuclear extracts of U2OS cells in untreated conditions and in response to 5 µM ICRF-193 treatment for 6 hours. The extract was diluted to the desired amount to reduce the amount of NaCl to 200 mM with 25 mM Tris-HCl-0.5 mM EDTA. The sample was centrifuged to remove protein precipitates that can occur at low salt. The protein extract was treated with 50 µg/ml ethidium bromide (Biorad) to disrupt DNA structure. This step is essential to avoid false positive interactions coming from the proteins being associated to the same DNA fragment.

Dynabeads® Protein G (Life Technologies) were washed with BC200 buffer (200 mM NaCl, 25 mM Tris-HCl and 0.5 mM EDTA) and blocked with 0.5 µg/µl BSA. Blocked magnetic beads were incubated for 30 minutes at 4ºC with the desired antibodies. After washing the beads 5 times to remove unbound antibodies, the extract was incubated with the magnetic beads for 16 hours at 4ºC. The beads were further washed 5 times with BC200 buffer containing 0.05% NP-40 (Sigma). Finally, the proteins associated with the magnetic beads were eluted with 1X NuPage loading buffer containing 12.5 mM 1,4-Dithiothreitol (DTT, Sigma) by incubating the beads at 70ºC for 10 minutes.

RESULTS

1 – Roles of NSMCE2 in genome maintenance and replication

1.1 - NSMCE2 recruitment to inter- and intra-strand crosslinks

In order to understand the functions of the SMC5/6 complex in mammals, we focused our studies on the SUMO ligase NSMCE2, which is an integral part of the SMC5/6 complex. NSMCE2 holds one of the catalytic activities of the complex and it was shown to be essential in yeast. With the use of various hypomorhic mutants, NSMCE2 (Mms21 in *Saccharomyces cerevisiae*) was found to participate in the processing of recombinogenic structures induced by the treatment with alkylating agents (Branzei *et al*., 2006). To investigate whether a similar response operates in mammals, wild-type MEFs were treated for one hour with MMS and let to recover for 24 hours in the absence of the drug. Exposure to MMS promoted the accumulation of NSMCE2 foci, supporting the role of the SMC5/6 complex in dealing with alkylating agents-induced damage **(Figure 1)**. Noteworthy, MMS-induced NSMCE2 foci in MEFs localized preferentially to pericentric heterochromatin, consistent with a preferential role of the SMC5/6 complex in the maintenance of repeated sequences. While NSMCE2 foci colocalized with H2AX phosphorylation (γH2AX) foci in response to MMS, the colocalization was not that evident in response to IR, which induced much fewer NSMCE2 foci, suggesting that the lesions recognized by NSMCE2 in response to MMS are distinct from DSBs.

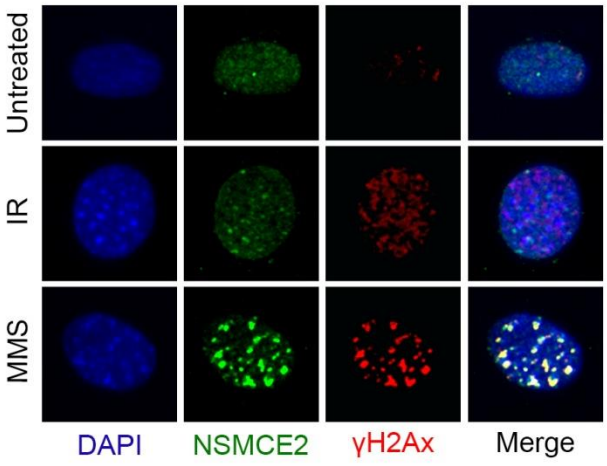

Fig. 1 - NSMCE2 foci in response to IR and MMS.

IR- (10 Gy and 1 hour recovery) and MMS- (1 mM for 1 hour and 24 hour recovery) induced NSMCE2 and γH2AX foci in MEFs. Upon IR, low intensity foci of NSMCE2 are observed and these accumulations do not colocalize with γH2AX. In response to MMS, NSMCE2 forms heterochromatic foci colocalizing with γH2AX. Scale bar indicates 5 μm.

Another strategy to assess the recruitment of proteins to sites of DNA damage is based on the use of a laser-microdissector. The advantage of using this approach resides in the possibility to select the region where damage is induced so that the recruitment of the desired protein can be easily visualised. When a UV-A laser is used in combination with a psoralen treatment, this method can be used for the local generation of ICLs. Psoralen and angelicin are able to intercalate in the double helix forming a covalent bond with a DNA strand when stimulated by a photon reaction. Since psoralen contains two photochemically reactive sites can be used to induce ICLs. Angelicin contains only one reactive site and, therefore, it will create mono-adducts upon UV-A stimulation.

To perform localized ICL experiments, I visited the laboratory of Dr. John Rouse at the University of Dundee where these experiments were conducted. By using this technology we were able to detect the recruitment of NSMCE2 to sites of UV-A damage in cells treated either with psoralen or angelicin (**Figure 2**), providing the first direct demonstration of the recruitment of the SMC5/6 complex to ICLs.

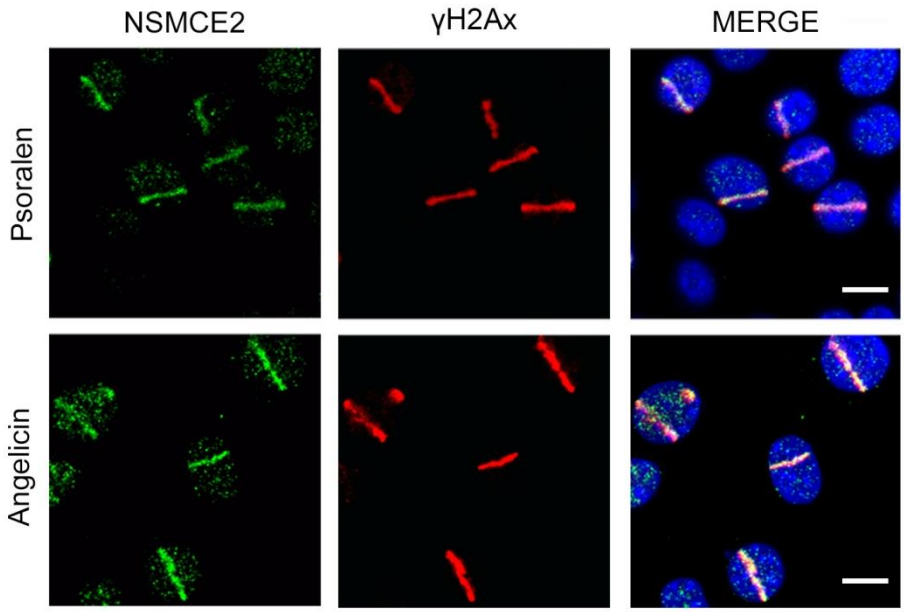

Fig. 2 - NSMCE2 recruitment to laser-induced ICLs.

A UV-A coupled laser microdissector was used in combination with 50 µM psoralen for 1 hour or 50 µM angelicin for 1 hour to evaluate the recruitment of NSMCE2 to sites of inter- or intra-strand crosslinks. Recruitment was assessed 30 minutes after inducing the damage. Scale bar indicates 10 µm. γH2AX was used to mark the sites of damage.

1.2 - NSMCE2 suppresses recombination and facilitates chromosome segregation

Our next aim was to evaluate the consequences of deleting *Nsmce2* in mammalian cells and organisms. To this end, a conditional knockout strain was generated (Jacome *et al*., 2015). When combined with a strain that expresses a tamoxifen inducible version of the Cre recombinase from the ubiquitin promoter (Ub.Cre^{ERT2}), this system allows for the controlled depletion of NSMCE2 **(Figure 3a)**. The first observation, consistent with the essential role of all SMC5/6 complex members, was that loss of NSMCE2 in MEFs severely impaired cell growth as demonstrated by clonogenic assays **(Figure 3b)**. To investigate the causes of the reduced viability of NSMCE2-deficient cells, Ub.Cre^{ERT2} *Nsmce2*$^{lox/lox}$ MEFs were plated in presence and without 4-OHT and cultured for 8 days. Nuclei morphology was then evaluated by DAPI staining. Loss of NSMCE2 protein resulted in a variety of nuclear abnormalities indicative of segregation problems with micronuclei being the most represented **(Figure 3c-3d)**. We also detected cells presenting highly irregular and multilobulated nuclei, which are likely the consequence of cumulative segregation problems during serial passaging. Of note, Cre expression in mammalian cells has been previously shown to slow down proliferation due to the fact that it creates DNA breaks (Loonstra *et al*., 2001). To discard the possibility that our phenotypes were influenced by the endonuclease activity of Cre, Ub.Cre^{ERT2} *Nsmce2*$^{+/+}$ MEFs were also included in the study. The presence of the Cre recombinase within the nucleus only minimally increased the percentage of segregation defects and did not visibly impair proliferation, confirming that the observed effects were due to NSMCE2 deficiency. Of note, NSMCE2 depletion did not affect the levels of chromatin-bound SMC5, suggesting that an NSMCE2-devoid SMC5/6 complex is not functional even if recruited to chromatin **(Figure 4)**.

Problems in chromosome segregation can arise either from true mitotic problems, but also from deficient DNA replication and repair which can lead to the presence of unreplicated regions of the genome reaching mitosis (Mankouri *et al*., 2013). The resolution of joint DNA molecules that reach mitosis is normally carried over by a pathway involving the BLM helicase, known as dissolution, and which does not involve DNA breakage (Mankouri *et al*., 2013, Mankouri *et al*., 2011). When dissolution fails and joint DNA molecules persist, a nuclease-dependent pathway known as resolution takes place, with the risk of genomic rearrangements. This is best exemplified by the large increase in sister chromatid exchanges (SCE) present in BLM-deficient cells. Interestingly, NSMCE2-deficient MEFs presented a significant increase in SCE levels **(Figure 3e-3f)**, indicating that its function could be similar to that of BLM, and suggesting that deficiencies in DNA replication and/or in the resolution of unresolved loci in mitosis could underlie the observed segregation defects. In support of this,

combined deletion of *Blm* and *Nsmce2* is lethal in mouse B lymphocytes (Jacome *et al.*, 2015).

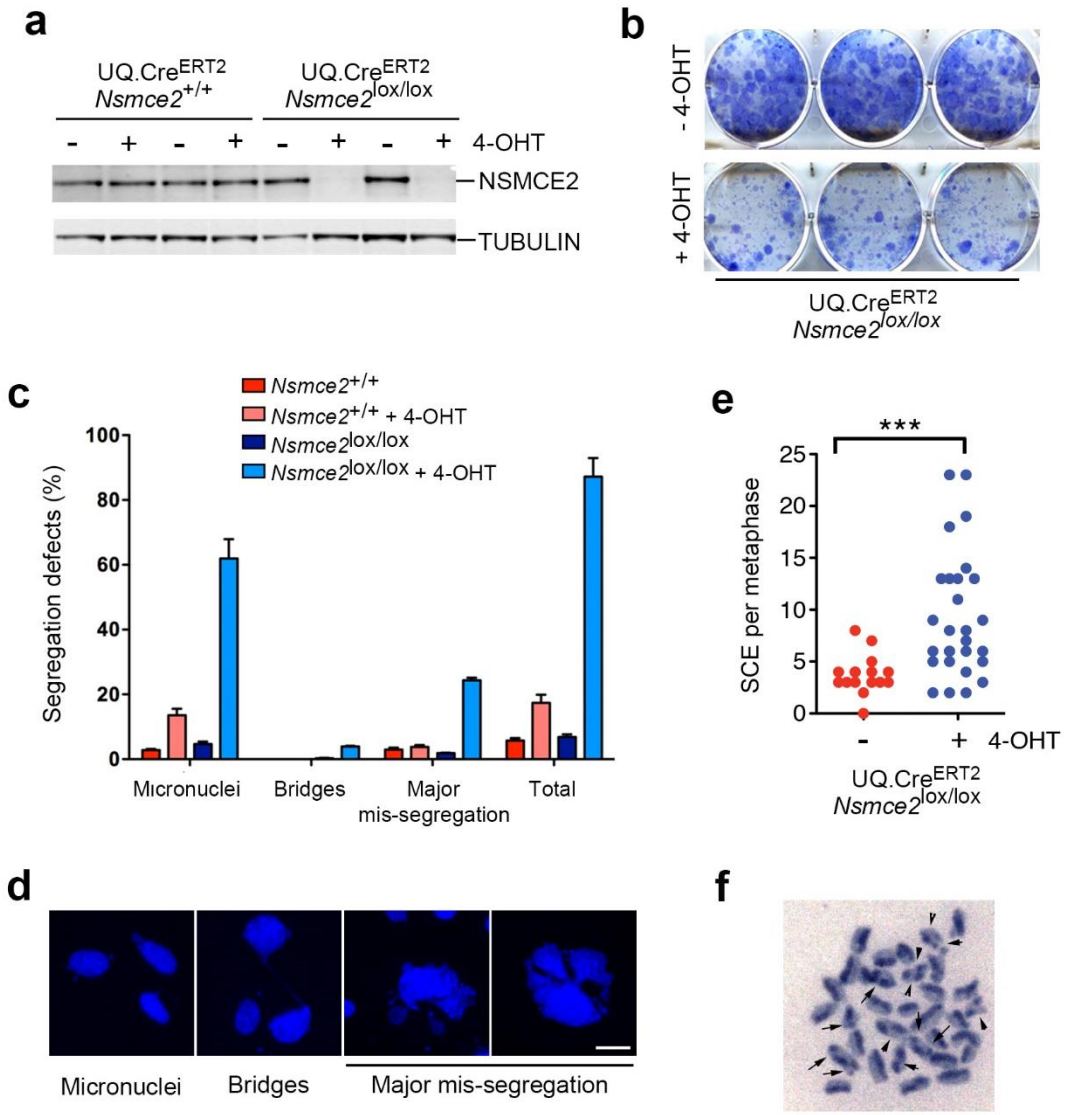

Fig. 3 - Defects observed in NSMCE2-deficient cells. Modified from Jacome *et al.* (2015).

a) Deletion of NSMCE2 assessed by Western blot (WB) in UQ.Cre^{ERT2} *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ MEFs after 48 hours of 4-OHT treatment. Tubulin levels are shown as a loading control.

b) Clonogenic assay on immortalized UQ.Cre^{ERT2} *Nsmce2*$^{lox/lox}$ MEFs. The picture shows the colonies detected after 10 days of growth in the presence or absence of 4-OHT, upon staining with crystal violet.

c) Quantification of the various segregation defects that can be observed by DAPI staining in UQ.Cre^{ERT2} *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ MEFs treated or not with 4-OHT for 8 days.

d) Examples of segregation defects observed in NSMCE2-deficient MEFs described in c). Scale bar indicates 2.5 µm.

e) SCE events per metaphase on UQ.Cre^{ERT2}/*Nsmce2*$^{lox/lox}$ MEFs grown in the presence of 4-OHT for 72 hours. The *P*-value was calculated with unpaired two-tailed t-test. *** $P<0.001$.

f) Example of a metaphase with increased levels of SCE due to NSMCE2 loss in MEFs mentioned in e). Arrows show the exchange of genetic material between sister chromatids.

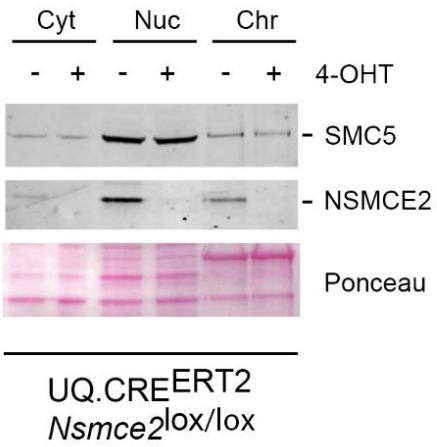

Fig. 4 – Localization of SMC5 is not affected by NSMCE2 depletion.

Cellular fractionation of Ub.Cre^{ERT2} *Nsmce2*$^{lox/lox}$ MEFs with and without 4-OHT treatment. Levels of SMC5 in the three fractions are not altered by NSMCE2 depletion.

1.3 - NSMCE2 is not essential for overall DNA replication in mammalian cells

To determine whether NSMCE2 deficiency has a direct effect on DNA replication, DNA fiber analysis was carried out in B-lymphocytes where *Nsmce2* was deleted by a Cre recombinase system knocked-in at the B-cell specific CD19 promoter **(Figure 5a)**. Since splenic B-cells are arrested in G_0, this system allows to evaluate the effects of NSMCE2 deficiency in the first cell cycle, eliminating the potential damage that could derive from previous cell cycles.

B-lymphocytes were stained with propidium iodide (PI) and subsequently analyzed by Flow cytometry to evaluate their cell cycle progression. As expected, G1 and G2 peaks were visible in these cells 48 hours after being stimulated *in vitro* with LPS. Interestingly, cultures of *Nsmce2* deleted B-cells presented cells with a >4n amount of DNA, further indicative of the segregation defect that arise in the context of NSMCE2 deficiency **(Figure 5b)**. DNA fiber analyses performed in these cells revealed that DNA replication was not significantly altered by NSMCE2 loss, neither in terms of origin firing, nor on fork rates **(Figure 5c-5d-5e)**. While we cannot exclude that NSMCE2 might play a specific role in the replication of certain loci (e.g. pericentromeric DNA or repeat regions), these analyses reveal that overall DNA replication is not affected by NSMCE2 deficiency in mammalian cells.

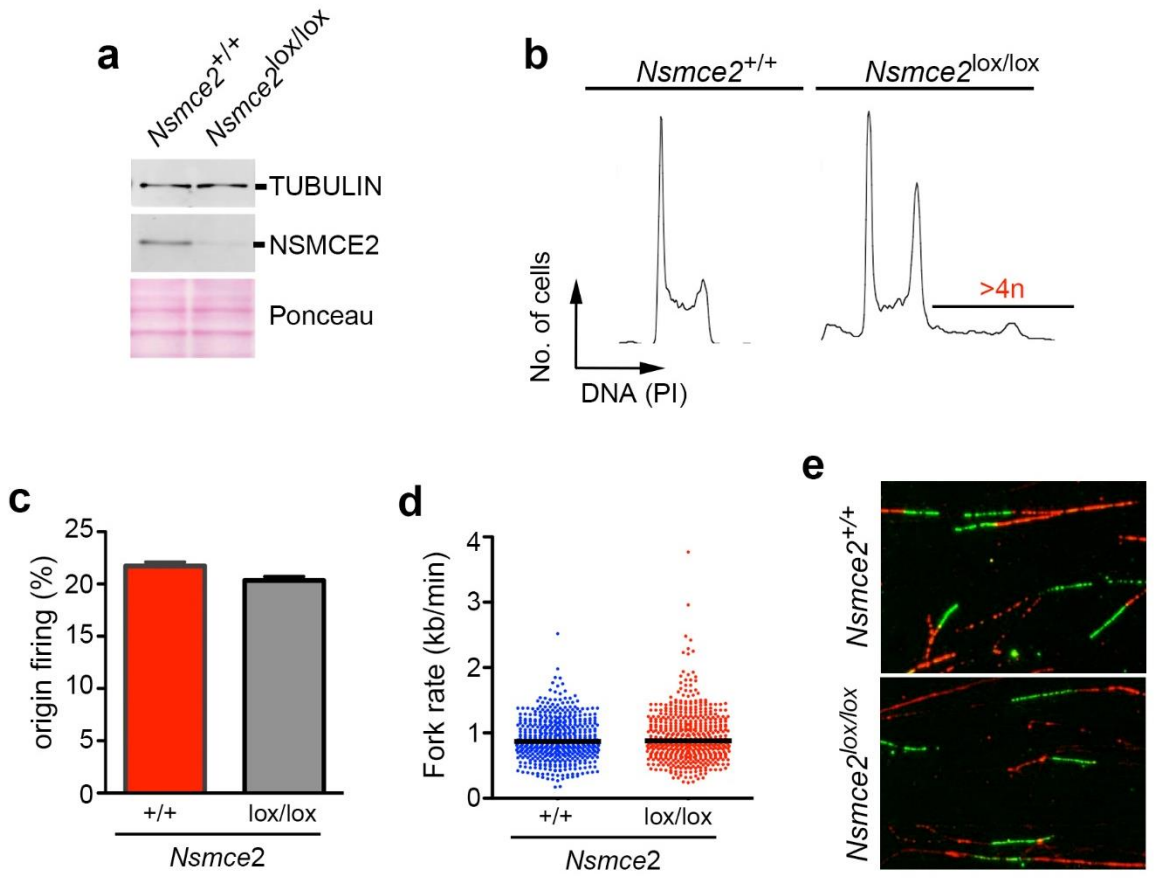

Fig. 5 - NSMCE2 is dispensable for DNA replication (Jacome *et al.*, 2015).

a) WB illustrating the depletion of NSMCE2 in B-lymphocytes isolated from 2 months old CD19$^{+/Cre}$ animals of the indicated genotypes, 48 hours after being stimulated *in vitro* with LPS. Tubulin was used as a loading control.

b) Representative flow cytometry analysis from the B-lymphocytes cultures mentioned in (a). DNA content was measured by PI staining. Note the presence of cells with > 4*n* DNA content in NSMCE2-deficient cells.

c-d) Frequency of origin firing (c) and fork rate (d) were analysed by DNA fiber analyses from fibers prepared from B-cell cultures explained in (a). Between 200 and 300 tracks were measured to estimate fork rate and 300–500 for the calculation of origin firing frequencies. No significant differences were observed between CD19$^{+/Cre}$ *Nsmce2*$^{+/+}$ and CD19$^{+/Cre}$ *Nsmce2*$^{lox/lox}$ B-cells.

e) Representative images of the DNA fibers used for the analysis explained in (c, d). CldU (red) and IdU (green) channels are shown.

1.4 - NSMCE2 suppresses ageing in mice independently of its SUMO ligase activity

Finally, and since NSMCE2 is essential for embryonic development, we evaluated the physiological impact of deleting *Nsmce2* in adult mice. To this end, UQ.Cre^{ERT2} *Nsmce2*$^{lox/lox}$ mice were fed with a 4-OHT containing diet starting at weaning. Deletion of the *Nsmce2* in adult mice led to a progressive and generalised progeroid syndrome **(Figure 6a-6b)**. As examples of the overall ageing process, NSMCE2-deficient mice presented premature hair greying and reduced body fat **(Figure 6c)**. In addition to the remarkable ageing phenotype, these animals presented other hallmarks of genomic instability syndromes such as Bloom's Syndrome or Fanconi anemia, including an altered pigmentation of the skin and progressive anemia **(Figure 6d)**.

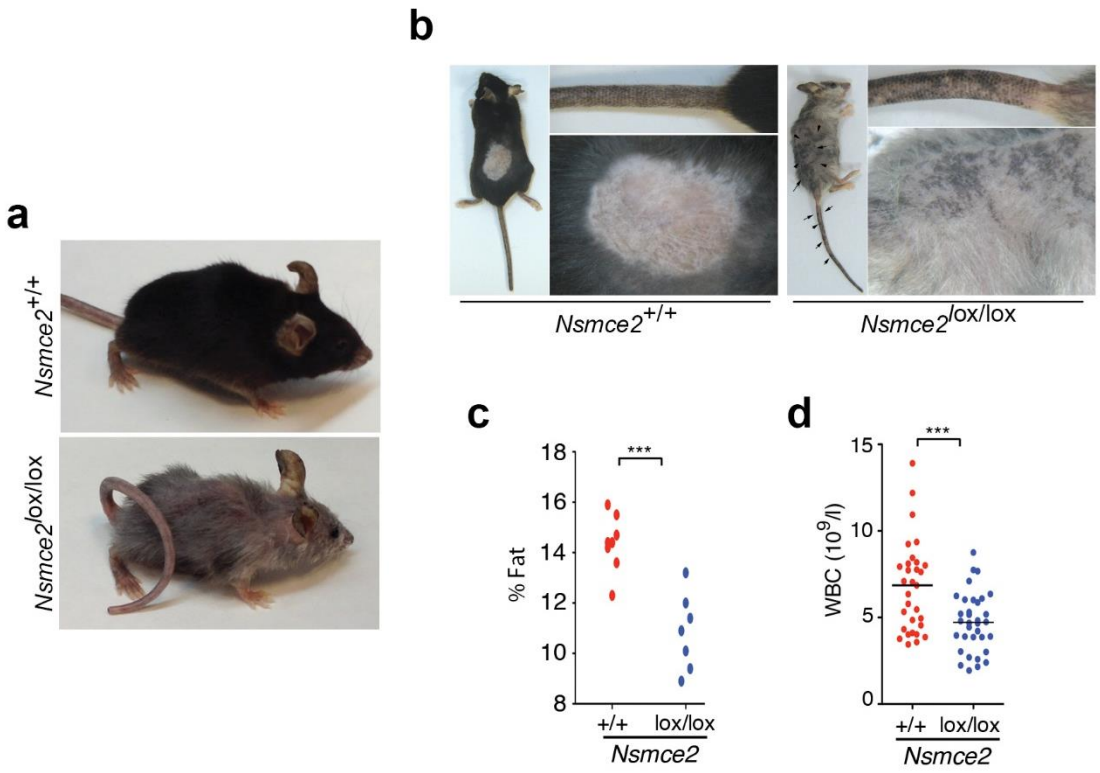

Fig. 6 - Impact of *Nsmce2* deletion in adult mice. Modified from Jacome *et al.* (2015).

a) UQ.Cre^{ERT2} *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ animals fed for 9 months with a 4-OHT-containing diet.

b) Altered pigmentation of the skin in UQ.Cre^{ERT2} *Nsmce2*$^{lox/lox}$ mice.

c) Percentage of fat against total body mass on *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ animals. The *P*-value was calculated with unpaired two-tailed t-test. *** *P*<0.001.

d) White blood cell counts (WBC) from *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ animals. The *P*-value was calculated with unpaired two-tailed t-test. *** *P*<0.001.

To evaluate the contribution of the SUMO ligase activity to the phenotype observed in *vivo*, mice lacking of NSMCE2 SUMO ligase activity were generated in our lab (Jacome *et al*., 2015). Loss of the SUMO ligase activity was achieved by using a knock-in mutant allele in which C185S and H187A mutations were introduced on the SP-RING domain (*Nsmce2*^{SD}). In contrast to NSMCE2 deficiency, loss of its SUMO ligase activity did not lead to accelerating ageing nor to any other apparent phenotype in mice **(Figure 7a)**. Furthermore, *Nsmce2*$^{SD/SD}$ animals were viable and fertile and showed no alteration of their lifespan **(Figure 7b)**. Inactivation of its SUMO ligase also did not affect the recruitment of NSMCE2 to MMS- or MMC-induced foci **(Figure 7c-7d)**.

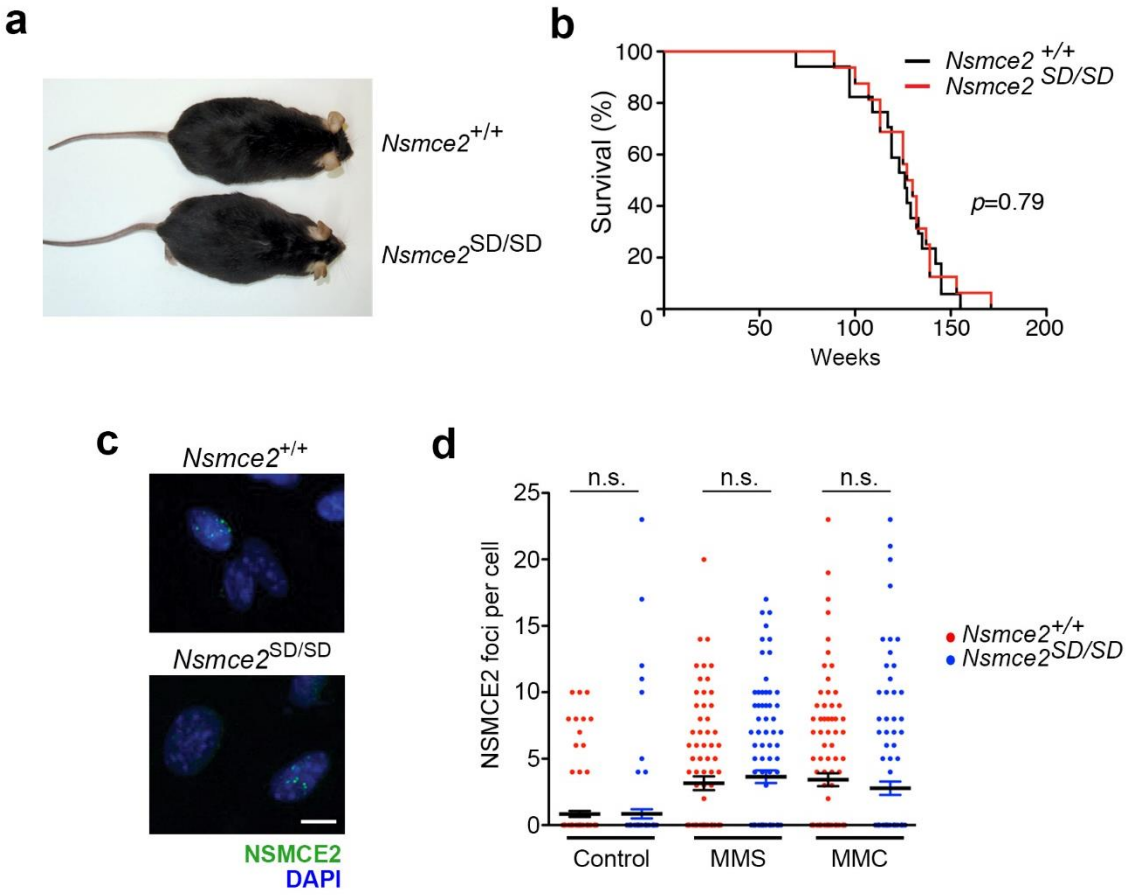

Fig. 7 - Abrogation of NSMCE2 SUMO ligase activity has no effect on mice physiology. Modified from Jacome *et al.* (2015).

a) Representative picture of 6 months old *Nsmce2*$^{+/+}$ and *Nsmce2*$^{SD/SD}$ mice.

b) Survival curves of *Nsmce2*$^{+/+}$ and *Nsmce2*$^{SD/SD}$ mice. The *P*-value was calculated with the Mantel–Cox long-rank test.

c) NSMCE2 foci in MMS-treated *Nsmce2*$^{+/+}$ and *Nsmce2*$^{SD/SD}$ MEFs. Scale bar indicates 2.5 µm.

d) High-Throughput Microscopy (HTM) mediated quantification of the number of NSMCE2 foci per cell in wild-type and *Nsmce2*$^{SD/SD}$ MEFs in response to MMS (1 mM for 1 hour and 16 hour recovery) or MMC (0.25 µM for 16 hours).

In summary, in this first part of the PhD we revealed that NSMCE2 suppresses recombination and segregation problems in mammalian cells, and performed a first general characterization of the impact of NSMCE2 deficiency in an adult organism.

2 - NSMCE2 deficiency in mice leads to karyomegalic interstitial nephritis.

2.1 - Post-natal *Nsmce2* deletion leads to Karyomegalic Interstitial Nephritis

Karyomegalic interstitial nephritis (KIN) is a late-onset degenerative disease of the kidney, in which giant nuclei progressively accumulate in the cortex until kidney dysfunction ensues (OMIM 614817). The discovery that mutations in *FAN1*, a nuclease involved in ICL repair (Zhou *et al*., 2012, Smogorzewska *et al*., 2010) are a cause oh hereditary KIN in humans, indicated that DNA damage accumulation could underlie the ontogeny of this disease (Zhou *et al.*, 2012). Later work confirmed the role of FAN1, as FAN1 deficiency in mice leads to a mild form of KIN (Lachaud *et al*., 2016, Thongthip *et al*., 2016). To date, *FAN1* is the only gene that has been identified as a cause of KIN.

Besides the overall progeria that is observed in NSMCE2-deficient adult mice, a careful analysis of the different organs revealed a distinct phenotype on their kidneys that worsened as the mice age. Macroscopically, kidneys of NSMCE2-deficient mice were smaller and of a lighter colour compared to the ones isolated from wild-type littermates **(Figure 8a)**. This pathology tended to proceed asymmetrically, with one of the kidneys ultimately reducing its size **(Figure 8b).** Renal histology through Hematoxylin-Eosin staining revealed the hallmark of KIN, which is the presence of karyomegalic cells in the renal cortex of NSMCE2-deficient animals **(Figure 8d)**. Quantification of the nuclear area of kidney cells confirmed the presence of larger nuclei in the cortex of NSMCE2-deficient kidneys, where nuclear size could reach up to 200 μm^2 **(Figure 8e)**. This peculiar alteration occurs specifically in the epithelial cells forming the renal tubuli of the cortex, whereas no major alteration in nuclear size was found in the renal medulla. Together with the presence of giant nuclei, the entire kidney architecture of NSMCE2-deficient mice appears disorganized, with irregular nuclei and areas of severe tubuli dilation **(Figure 8c-8d)**. As observed in human KIN patients, the kidneys of NSMCE2-deficient mice presented a progressive accumulation of fibrotic tissue as detected by a Masson's Trichrome-staining **(Figure 8f)**. Noteworthy, and in contrast to what was reported in the kidneys of FAN1-deficient mice (Lachaud *et al*., 2016), we failed to detect γH2AX positive staining in the enlarged tubular cells observed on NSMCE2-deficient kidneys **(Figure 8g)**. The onset of the KIN phenotype (as well as the progeria) varies from mouse to mouse likely reflecting the variability on *Nsmce2* deletion that occurs with the 4-OHT-containing diet.

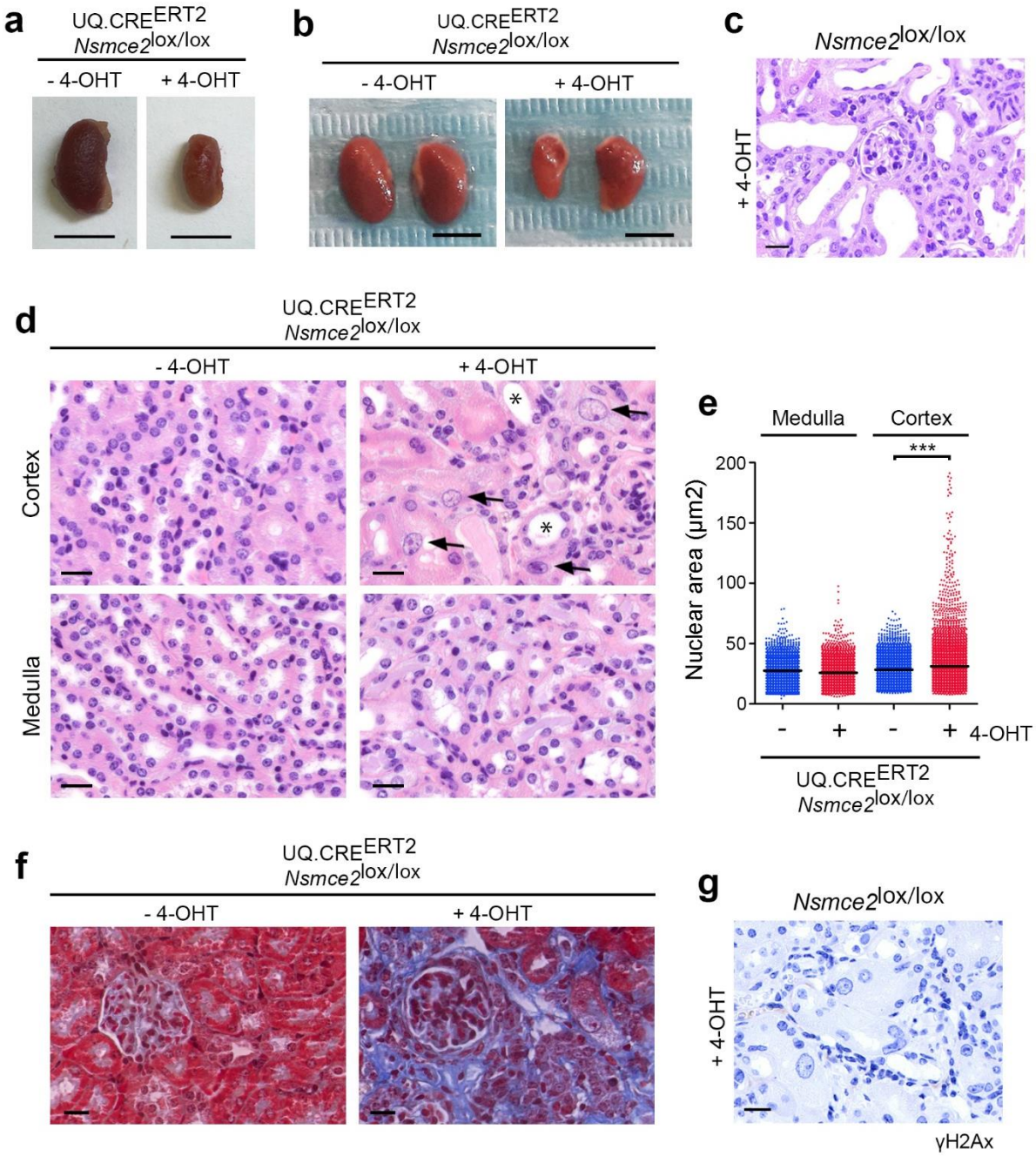

Fig. 8 - Hallmarks of KIN in *Nsmce2* conditional knockout mice.

a) Kidneys from 9 months old UQ. Cre^{ERT2} *Nsmce2*$^{+/+}$ and UQ. Cre^{ERT2} *Nsmce2*$^{lox/lox}$. Scale bar indicates 5 mm.

b) Representative example of kidney shrinkage observed in a 12 months old UQ. Cre^{ERT2} *Nsmce2*$^{lox/lox}$ mice on a 4-OHT diet. Scale bar indicates 5 mm.

c) Severe tubular dilation observed in UQ. Cre^{ERT2} *Nsmce2*$^{lox/lox}$ mice on a 4-OHT diet. Scale bars indicates 20 µm.

d) Histological analysis by hematoxylin-eosin staining of kidneys from *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ mice, 10 months after being fed with a 4-OHT containing diet. Cells in renal tubular epithelia (black arrow) and partial dilation of renal tubules (asterics) are observed. Scale bars indicates 20 µm.

e) Quantification of nuclear area in the different renal compartments. The *P*-value was calculated with unpaired two-tailed t-test. *** *P*<0.0001

f) Analysis of fibrosis (in blue) by Masson's Trichrome staining in renal sections from wild-type and UQ. Cre^{ERT2} *Nsmce2*$^{lox/lox}$ mice 10 months after being fed with a 4-OHT containing diet. Scale bars 20 µm.

g) γH2AX staining in kidneys of 10 months old UQ. Cre^{ERT2} *Nsmce2*$^{lox/lox}$ mice fed with 4-OHT diet.

In order to evaluate whether the KIN phenotype compromised the physiology of the organ, blood analyses were performed looking at markers of kidney function. To this end, levels of urea, creatinine and albumin were analysed in blood serum. Urea and creatinine are internal waste metabolites that need to be expelled into the urine through the kidneys. If kidney function is impaired, these compounds are not properly filtered and their blood levels rise. Indeed, NSMCE2-deficient mice present higher urea and creatinine levels in blood compared to wild-type mice, indicating that kidney function is compromised in these mice **(Figure 9)**. In contrast to waste metabolites, albumin is a valuable compound that needs to be retained in the bloodstream. The size of the molecule prevents its filtration through the kidney. However, if glomeruli structure is disrupted and its filtration ability compromised, it is expelled into the urine resulting in a decrease of albumin in blood. Lower levels of albumin in the blood NSMCE2-deficient mice further confirmed renal dysfunction **(Figure 9)**. The low amount of urine that we could obtain (particularly from mutant animals), prevented an analogous analysis of the urine. Taken together, these data suggests indicate that NSMCE2 deficiency in adult mice leads to KIN, which ultimately compromises kidney function.

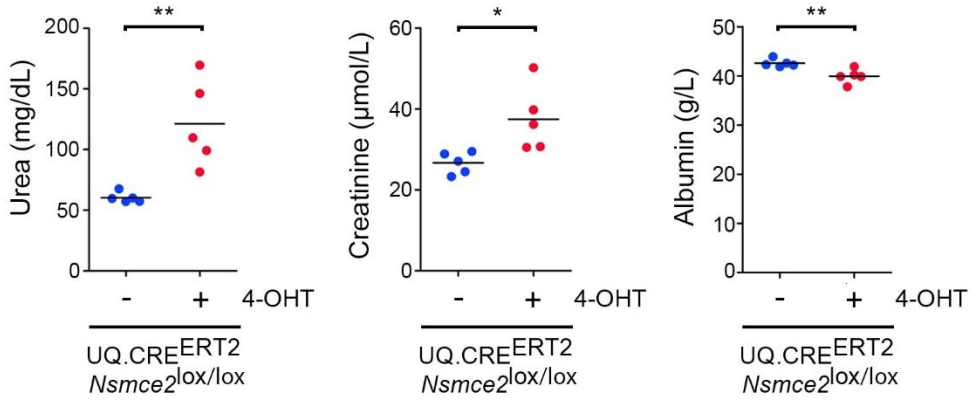

Fig. 9 - *Nsmce2* deletion in adult mice leads to kidney failure.

Urea, Creatinine and Albumin levels in blood serum of UQ. Cre^{ERT2} $Nsmce2^{+/+}$ and UQ. Cre^{ERT2} $Nsmce2^{lox/lox}$ between 50 and 55 weeks of age fed with 4-OHT containing diet starting at eaning. Each dot represents an independent mouse. The *P*-value was calculated with unpaired two-tailed t-test. *= $P<0.01$; **= $P<0.001$; *** $P<0.0001$.

2.2 - NSMCE2-dependent SUMOylation is dispensable for normal kidney function

In order to evaluate if the observed KIN was related to the SUMO ligase activity of NSMCE2, a careful analysis of the kidney was also performed in *Nsmce2*$^{SD/SD}$ mice. In contrast to what we observed in NSMCE2-deficient animals, kidney shape, size and colour did not differ between wild-type and SUMO-dead animals **(Figure 10a)**. In addition, immunohistochemistry analyses failed to detect karyomegalic cells, tubular dilation or fibrosis in the mutant kidneys (**Figure 10b-10c)**. In summary, loss of the SUMO ligase activity of NSMCE2 has no detectable impact on kidney physiology.

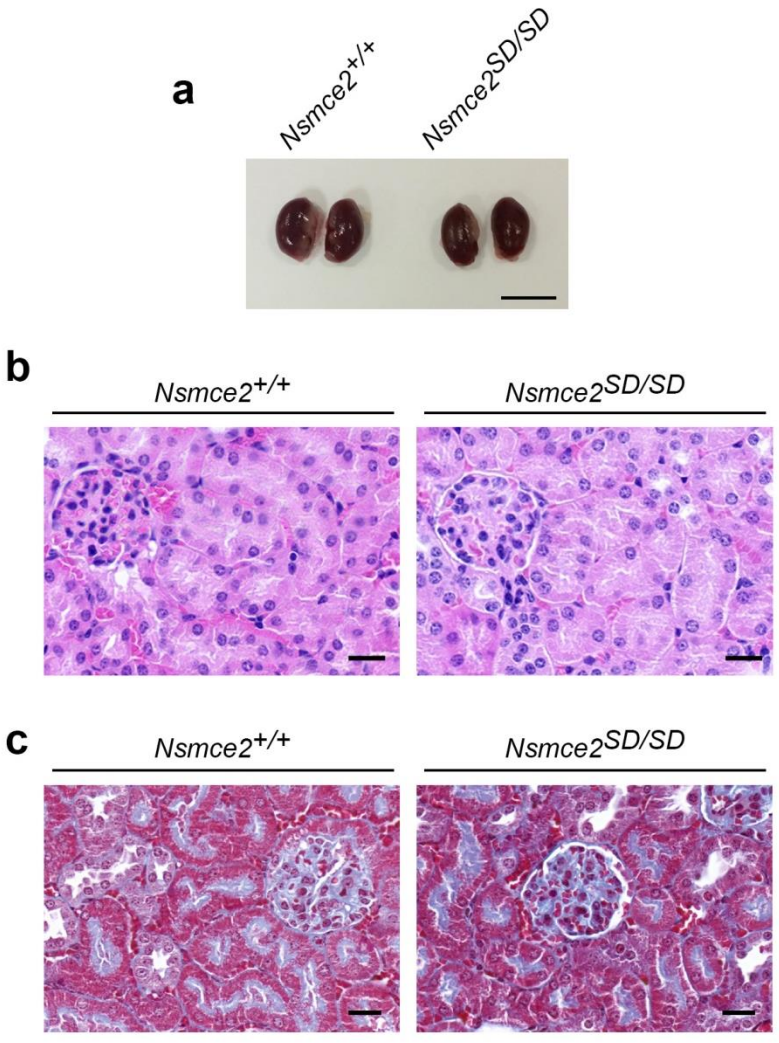

Fig. 10 - *Nsmce2*$^{SD/SD}$ mice have no signs of KIN.

a) Kidneys from 15 months old *Nsmce2*$^{+/+}$ and *Nsmce2*$^{SD/SD}$ mice. Scale bar indicates 1 cm.

b) Histological analysis by hematoxylin-eosin staining of renal cortex from 12 months old *Nsmce2*$^{+/+}$ and *Nsmce2*$^{SD/SD}$ mice. No karyomegalic cells were found. Scale bars indicates 20 µm.

c) Analysis of fibrosis (in blue) by Masson's Trichrome staining in renal sections from *Nsmce2*$^{+/+}$ and *Nsmce2*$^{SD/SD}$ mice. Scale bars indicates 20 µm.

2.3 - NSMCE2 is recruited to sites of topological stress

From clinical cases of KIN that have been reported in the literature, some of them have been associated with the exposure to ochratoxin A (OTA) (Hassen *et al*., 2004, Godin *et al*., 1996), a mycotoxin produced by several species of *Aspergillus* and *Penicillium* that is sometimes found as a food contaminant. The patients of KIN exposed to OTA presented all the hallmarks of the disease. In addition, OTA can be used to induce KIN experimentally in mice and rats (Adler *et al*., 2009, Vettorazzi *et al*., 2011, Taniai *et al*., 2014). However, for a long time the mechanism of action of OTA-induced nephrotoxicity was unknown. Finally, in 2009 Cosimi and colleagues demonstrated that OTA was a catalytic inhibitor of DNA topoisomerase II (TOPOII), although this study did not connect this finding to the ontogeny of KIN.

Abundant work from model organisms had already shown that the functions of the SMC5/6 complex are related to Topoisomerases (Kegel *et al*., 2011, Jeppsson *et al*., 2014). To investigate if mammalian NSMCE2 was responsive to topological stress we first evaluated the distribution of NSMCE2 after exposing cells to either Etoposide (a topoisomerase poison that generates breaks due to the trapping of an intermediate product) or ICRF-193 (a catalytic inhibitor of TOPOII) in mouse NIH-3T3 cells (Huang *et al*., 2001). Under these conditions, NSMCE2 is dramatically concentrated around pericentric heterochromatin, in foci that also contain γH2Ax (**Figure 11a)**. We should note that from all conditions we ever tested in the laboratory (including IR, HU, DNA repair inhibitors, etc…) to induce NSMCE2 foci, the catalytic inhibitor ICRF-193 is the one that promotes the most intense recruitment of NSMCE2 (**Figure 11b)**.

TOPOII inhibition and NSMCE2 deficiency also share a peculiar phenotype, the presence of diplochromosomes. Diplochromosomes consist of four chromatids held together and are the visual sign of mitotic defects. Although the true source of diplochromosomes is unknown, they are thought to be linked to endoreplication, namely two rounds of DNA replication without mitotic division and therefore, without chromatid separation (Sumner, 1998, Cortés and Pastor, 2003). One of the few conditions that has been shown to experimentally induce diplochromosomes in mammalian cells is the inhibition of TOPOII (Sumner, 1998, Ji *et al*., 2009, Cortés and Pastor, 2003). Interestingly, metaphase spreads from *Nsmce2* conditional knockout MEFs presented several diplochromosomes **(Figure 12a-12b)**, once again reinforcing the link between NSMCE2 and Topoisomerase activity. The visualization of these structures was facilitated by labelling the telomeres of each chromatid with distinct probes by CO-FISH staining, which was performed with the help of the group of Dr. Maria Blasco at the Spanish National Cancer Research Center.

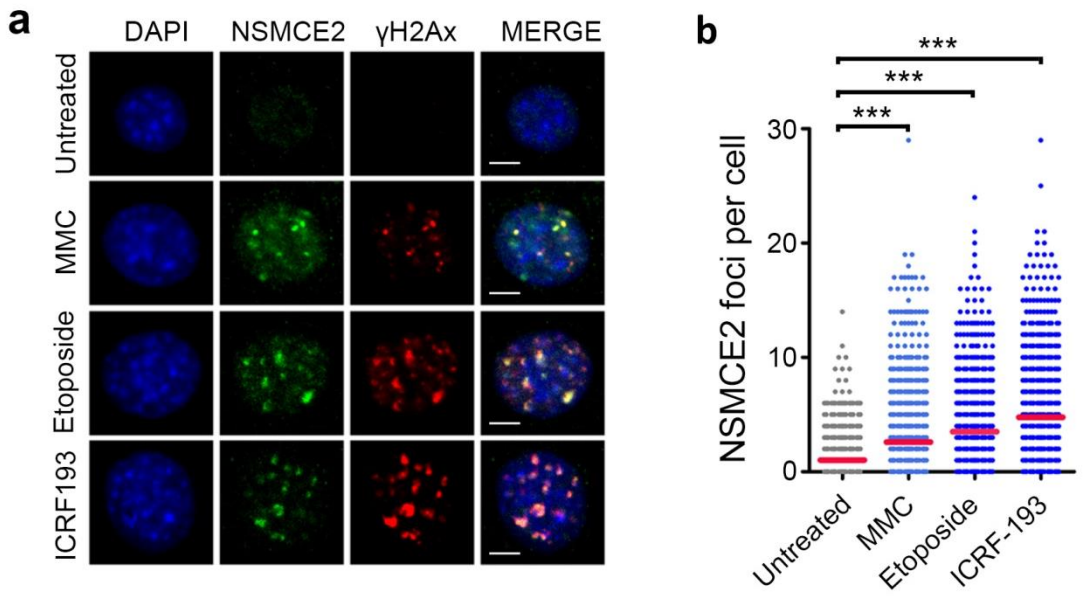

Fig. 11 - NSMCE2 is recruited to topological stress.

a) NSMCE2 foci colocalizing with γH2AX in 3T3 cells in response to MMC (300 nM for 6 hours), Etoposide (0.5 µM for 6 hours) and ICRF-193 (5 µM for 6 hours). Scale bar 5 µm.

b) Quantification of NSMCE2 foci per cell in 3T3 cells upon MMC, Etoposide and ICRF-193 treatment. The *P*-value was calculated with unpaired two-tailed t-test. *** *P*<0.0001

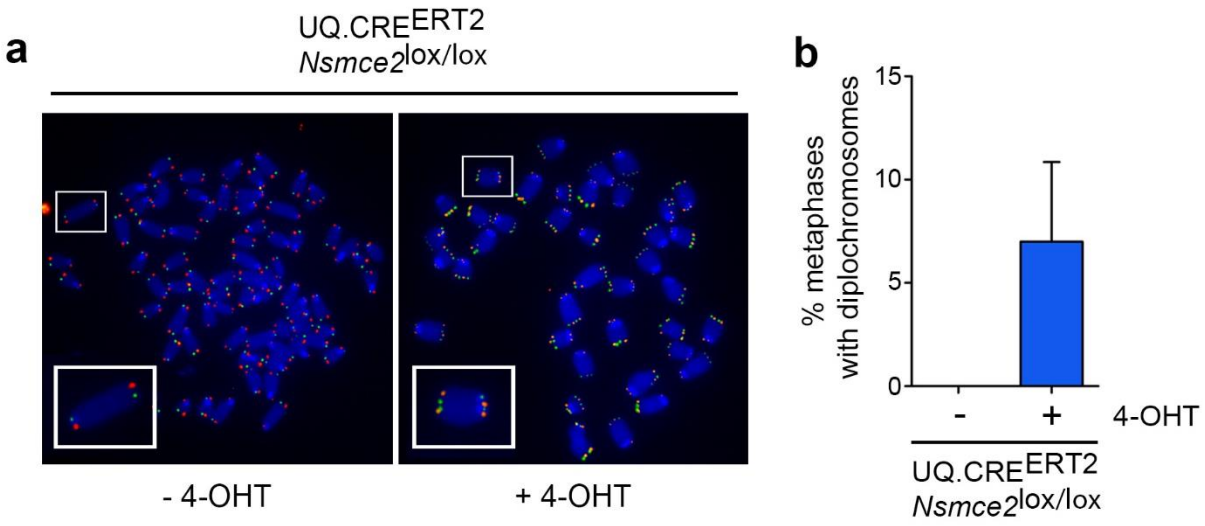

Fig. 12 - Loss of NSMCE2 leads to diplochromosomes.

a) Diplochromosomes are found in the metaphase spreads of UQ. Cre^{ERT2} $Nsmce2^{lox/lox}$ MEFs treated with 4-OHT for 48 hours to delete *Nsmce2*. For better visualization of these structures, CO-FISH was used to mark telomeric ends. The hybridization of a strand-specific telomeric probe allowed to distinguish the orientation of the chromatid.

b) Quantification of the metaphases characterized by the presence of diplochromosomes in cells mentioned in a).

2.4 - Response of NSMCE2-deficient cells to Topoisomerase inhibition

As mentioned, SMC5/6 deficiencies and TOPOII inhibition share similar phenotypes and are both involved in pathways of DNA topology maintenance. It is well known that their actions in yeasts are epistatic and their combined deficiency results in synthetic lethality due to severe segregation defects (Takahashi *et al*., 2008, Outwin *et al*., 2009, Jeppsson *et al*., 2014). In order to investigate the relationship between NSMCE2 and TOPOII in mammalian cells, we explored the sensitivity of NSMCE2-deficient cells to TOPOII poisons. First, we evaluated the response of $CD19^{+/Cre}$ $Nsmce2^{+/+}$ and $Nsmce2^{lox/lox}$ B-lymphocytes to the catalytic inhibition of TOPOII **(Figure 13a)**. Cell survival was assessed to a range of ICRF-193 concentrations by scoring DAPI-positive dead cells by flow cytometry. While ICRF-193 was toxic for B-cells, there was still no clear sensitivity of *Nsmce2* knockout cell to TOPOII inhibition. However, one limitation of this approach is that NSMCE2 deficiency had already a very severe effect on cell survival in B-cells, which could likely mask the effect of any additional treatment **(Figure 13b-13c)**.

Next, the sensitivity to ICRF-193 was assessed in *Nsmce2* conditional knockout MEFs by a CellTiter-Glo® Luminescent Cell Viability Assay, which measures mitochondrial activity. Like in the case of B-cells, NSMCE2 deficiency in MEFs resulted by itself in a clear reduction in proliferation and no clear additional impact of the drug was observed **(Figure 13d)**.

Since the cell-essential nature of NSMCE2 limited the analysis in terms of survival, we focused on analysis on segregation defects. While ICRF-193 treatment does not result in increased cell death, it aggravated the segregation defects present in *Nsmce2* mutant cells. For instance, flow cytometry analyses revealed the presence of a tetraploid peak in NSMCE2-deficient B-cells exposed to ICRF-193 **(Figure 14a-14b)**. In addition, NSMCE2 conditional knockout MEFs accumulated more DNA bridges than wild-type MEFs when treated with the TOPOII inhibitor **(Figure 14c)**. These bridges are positive for the H3K9m3, which by immunofluorescence in mouse cells it mostly marks pericentromeric chromatin, suggesting that these structures might arise from the missegregation of centromeric regions **(Figure 14d)**. Besides segregation problems, TOPOII inhibition also increased the number of chromosome breaks that were induced by ICRF-193 in NSMCE2-deficient B-cells **(Figure 15a-15b)**.

In summary, our data support that a key role of the SMC5/6 complex in the response to topological stress, exemplified by the recruitment of NSMCE2 to sites of TOPOII induced damage and that NSMCE2 deficiency phenocopies many of the effects of TOPO II inhibition.

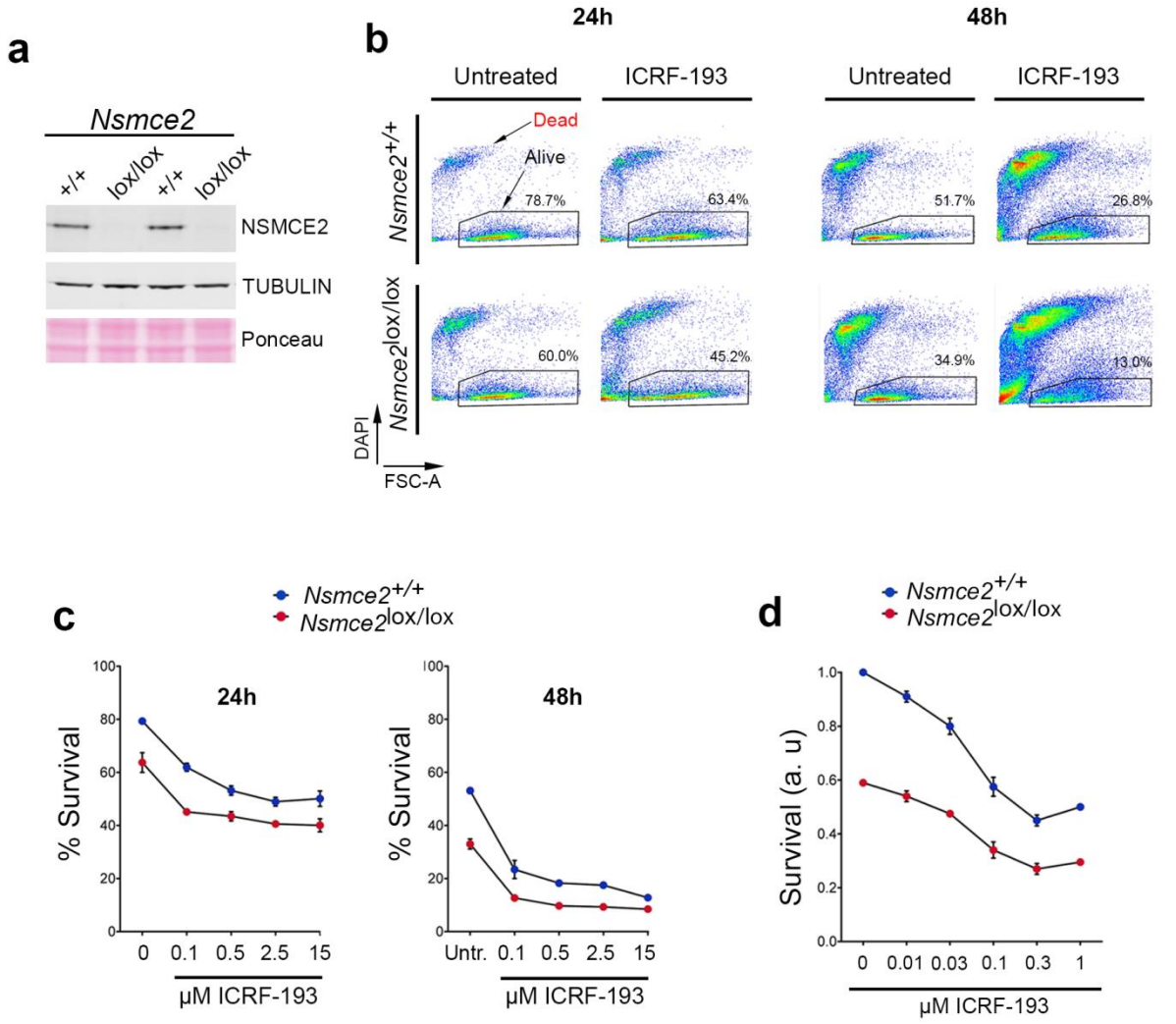

Fig. 13 - *Nsmce2* knockout cells are not more sensitive than wild-type cells to TOPOII inhibition.

a) WB illustrating the depletion of NSMCE2 in CD19$^{+/Cre}$ *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ B-cells, 48 hours after being stimulated *in vitro* with LPS. Tubulin was used as a loading control.

b) Viability assay by DAPI staining of CD19$^{+/Cre}$ *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ B-cells. Cells were stimulated with LPS for 24 hours followed by 24 and 48 hours treatment with a range of ICRF-193 concentrations (0-15 µM).

c) Percentages of cell survival, obtained by flow cytometry analysis measuring DAPI incorporation in CD19$^{+/Cre}$ *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ B-cells described in (a) exposed to a range of ICRF concentration (0-15 µM) for 24 and 48 hours.

d) Normalized survival from CellTiter-Glo® Luminescent Cell Viability Assay of UQ. Cre^{ERT2} *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ MEFs treated with 4-OHT for 48 hours to induce *Nsmce2* deletion, followed by ICRF-193 (0-1 µM) treatment for 48 hours.

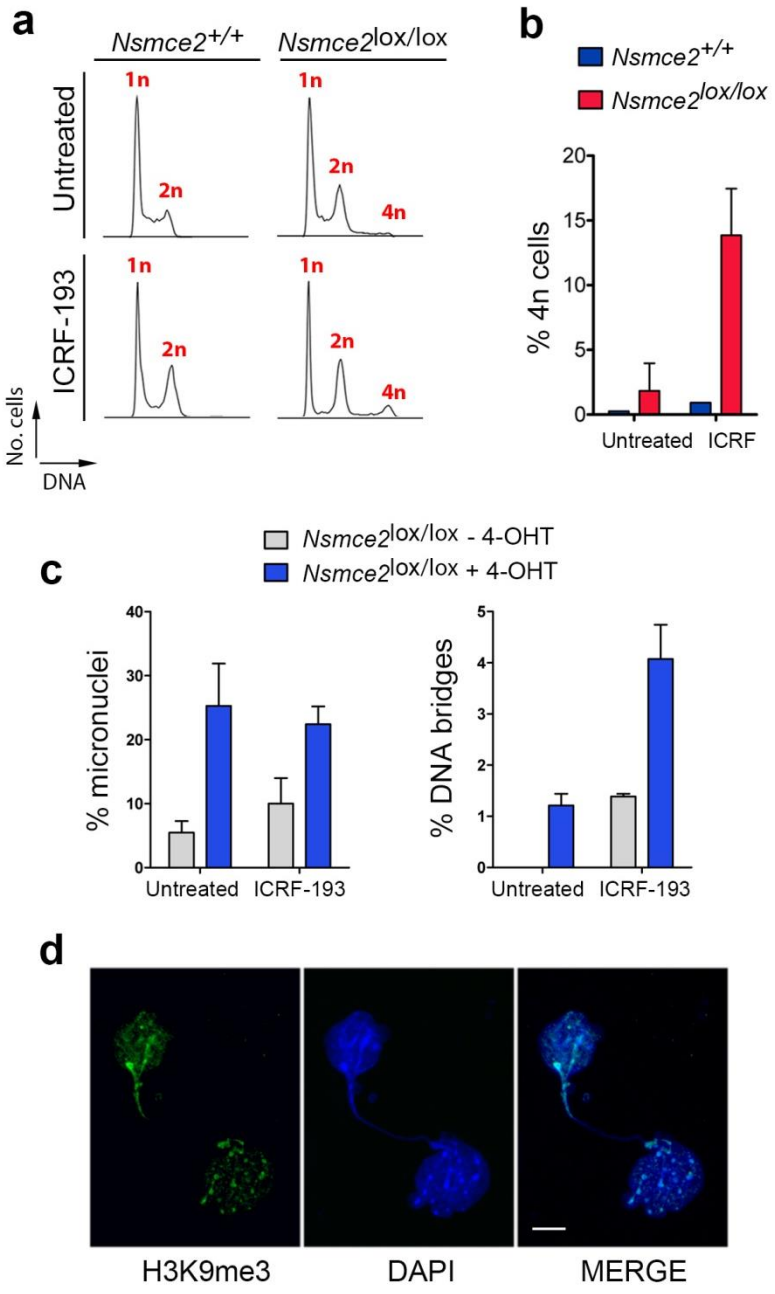

Fig. 14 - TOPOII inhibition and *Nsmce2* deletion impair chromosome segregation.

a) Representative flow cytometry analysis from the B-cell cultures. DNA content was measured with HOECHST staining. Note the presence of cells with >4n DNA content in NSMCE2-deficient B-cells treated with 0.1 µM ICRF-193 for 24 hours.

b) Quantification of cells with >4n DNA content in CD19$^{+/Cre}$ *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ B-cells mentioned in (a).

c) Percentage of micronuclei and DNA bridges in UQ. Cre^{ERT2} *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ MEFs treated with 4-OHT for 48 hours to induce *Nsmce2* deletion and with 0.5 µM ICRF-193 for the last 16 hours.

d) Representative ICRF-193-induced missegregation of centrometic regions marked for H3k9m3. Scale bar indicates 10 µm.

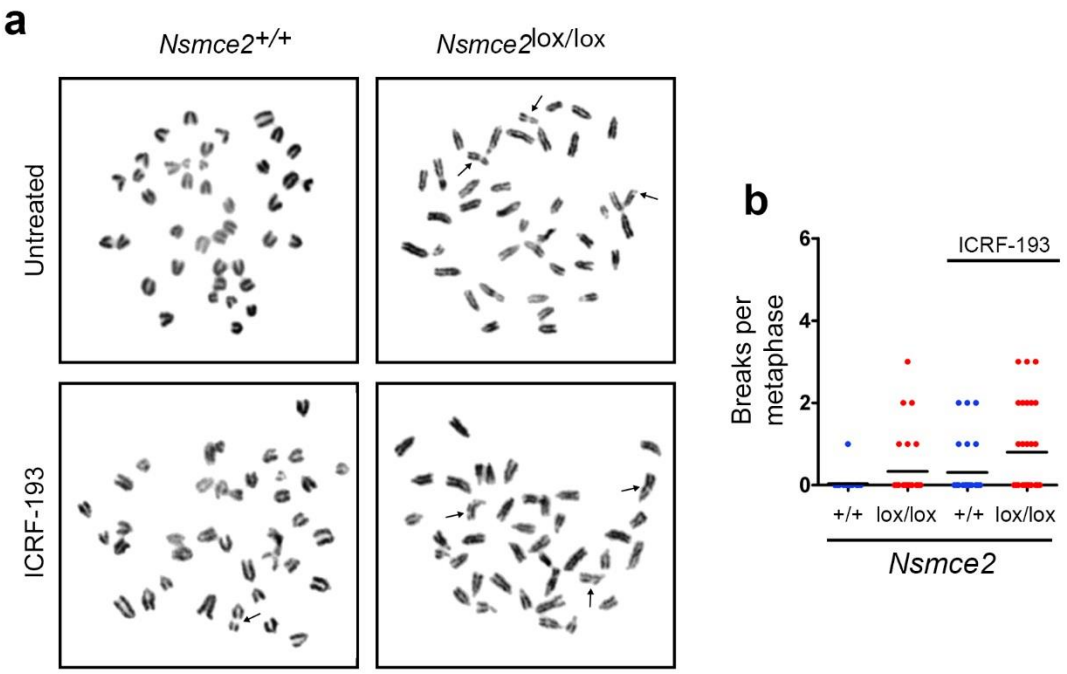

Fig. 15 - Chromosomal defects in NSMCE2-deficient cells treated with TOPOII poisons.

a) Metaphases spreads of CD19$^{+/Cre}$ *Nsmce2*$^{+/+}$ and *Nsmce2*$^{lox/lox}$ B-cells, stimulated for 48 hours with LPS and treated with 0.5 µM ICRF-193 for the last 16 hours. TOPOII inhibition in knockout cells results in increased chromosome breaks (arrows).

b) Quantification of number of breaks per metaphases mentioned in (a).

2.5 - NSMCE2 deficiency increases the levels of chromatin loaded TOPOII

Since the best indicator of topological stress could be the presence of Topoisomerases, we evaluated the levels of chromatin-bound TOPOII in *Nsmce2* conditional knockout MEFs by High-Throughput Microscopy (HTM). To enhance TOPOII recruitment, cells were also exposed to ICRF-193 for one hour. Interestingly, the levels of chromatin-bound TOPOII were constitutively higher in NSMCE2-deficient cells, a difference that was increased in the presence of ICRF-193 **(Figure 16a-16b)**. These data further support an important role of NSMCE2 in suppressing topological stress.

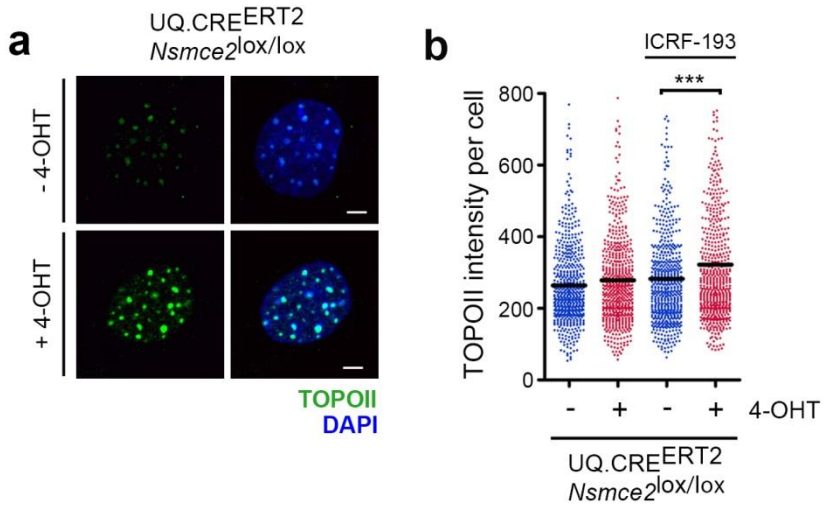

Fig. 16 - TOPOII accumulates at centromeres in *Nsmce2* conditional knockout cells.

a) Representative images of TOPOII foci intensities in UQ. Cre^{ERT2} $Nsmce2^{lox/lox}$ MEFs with and without 4-OHT treatment for 48 hours. Scale bar indicates 5 µm.

b) Quantification of TOPOII intensity in MEFs described in a) in untreated condition and upon treatment of 10 µM ICRF-193 for 1 hour.

2.6 - FAN1 and NSMCE2 are both involved in processing DNA topological stress

To date, the only mutation found in human patients of KIN is a mutation in a gene encoding the nuclease *FAN1* (Zhou *et al*., 2012). FAN1 was originally described to be part of the Fanconi Anemia (FA) pathway because of its direct interaction with ubiquitinated FANCD2, a key event of the FA pathway (Smogorzewska *et al*., 2010, MacKay *et al*., 2010). These

original studies placed FAN1 as a central player in ICL repair and thus in the FA pathway. However, human *FAN1* mutant patients do not present Fanconi Anemia (Trujillo *et al*., 2012), raising doubts on whether the true physiological role of FAN1 is on ICL repair.

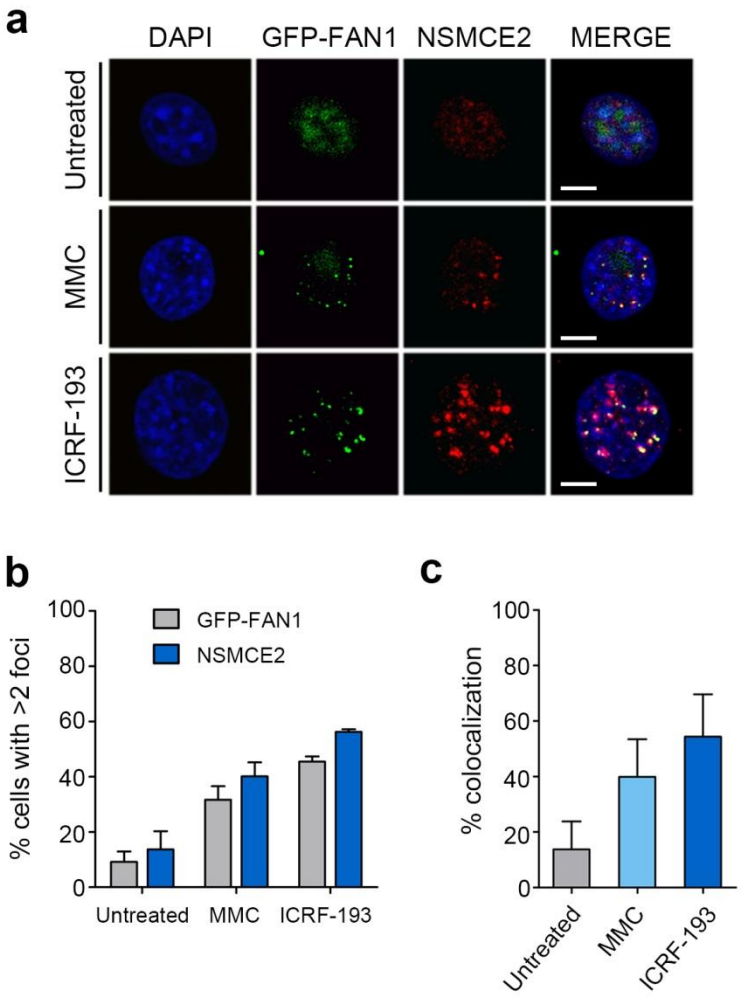

Fig. 17 - NSMCE2 and FAN1 are both involved in topological stress.

a) Colocalization of NSMCE2 and GFP-FAN1 foci in 3T3 cells treated in response to MMC (300 nM for 6 hours) and ICRF-193 (5 µM for 6 hours). Scale bar indicates 5 µm.

b) Quantification of cells with >2 NSMCE2 and FAN1 foci in the different treatments mentioned in (a).

c) Percentage of colocalization expressed as total number of cells with NSMCE2-FAN1 colocalization out of the total number of cells with >2 NSMCE2 foci.

Our data with NSMCE2-deficient mice, and the fact that the KIN induced agent OTA is a TOPOII catalytic inhibitor, led us to explore whether there could also be a connection between FAN1 and topological stress, and between FAN1 and NSMCE2. To this end, we first use a GFP-FAN1 construct that was transiently transfected in 3T3 cells. FAN1 foci were monitored after treatment with the TOPOII poison ICRF-193 and the ICL-inducing agent MMC. FAN1 formed foci in heterochromatin upon both treatments that colocalized with NSMCE2 foci **(Figure 17a)**. Of note, even though they colocalized, the two proteins presented different patterns of foci with FAN1 forming smaller accumulations. As in the case of NSMCE2, FAN1 foci formation was better induced by ICRF-treated cells than by MMC treated **(Figure 17b-17c)**. Whereas all current studies have focused on the role of FAN1 in ICL repair, our data presents the first evidences of a role for this protein in the response to topological stress.

2.7 - SMC5/6 complex does not physically interact with FAN1

Given the colocalization of FAN1 and NSMCE2, the potential physical interaction between FAN1 and the SMC5/6 complex was investigated. To this end, co-immunoprecipitation assays were carried out in U2OS cells in untreated condition, and after ICRF-193 treatment, to see if their potential interaction could take place exclusively upon TOPOII inhibition. As expected, the pull-down of SMC5 immunoprecipitated NSMCE2 **(Figure 18)**.

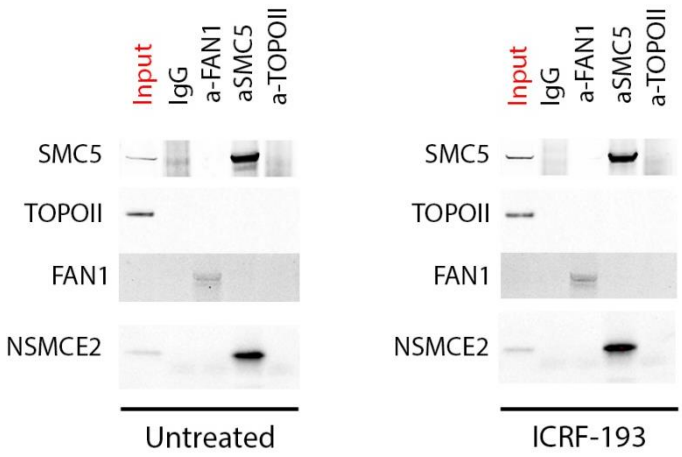

Fig. 18 - NSMCE2, FAN1 and TOPOII do not interact physically.

Co-immunoprecipitation assay in U2OS showing no physical interaction between SMC5, FAN1 and TOPOII in untreated condition (left panel) and upon 6 hours 5 µM ICRF-induced damage (right panel). Extracts were treated with ethidium bromide to avoid false positive interactions.

However, the SMC5/6 complex does not interact directly with FAN1, although sharing the same localization on chromatin. In addition, TOPOII was not found in SMC5 and FAN1 pull-downs. We shall note that another recent report has shown coimmunoprecipitation of TOP2A with SMC5/6 (Verver *et al*., 2016), but we have failed to see this interaction in our experimental conditions. Nevertheless, the absence of a physical interaction does not exclude that both complexes might be operating on a common substrate.

2.8 - FAN1 and NSMCE2 are independently recruited to the site of damage

We next investigated whether the recruitment of FAN1 and NSMCE2 is dependent on each other. First, we measured NSMCE2 recruitment in MEFs where *Fan1* was deleted using the CRISPR-Cas9 technology **(Figure 19a)**. These cells presented a lower number NSMCE2 foci in response to MMC and ICRF-193 treatment compared to wild-type cells **(Figure 19b-19c)**. Accordingly, γH2AX staining was also less intense **(Figure 19d)**. These data suggest that FAN1-deficient cells display reduced DNA damage by either affecting proliferation or by impairing the recruitment of DNA repair factors, such as NSMCE2, that are essential to boost the DNA damage response. At this point we must be cautious in the interpretation of these results, since the formation of foci can be influenced by replication rates. In any case, they do show that NSMCE2 can be recruited independently of FAN1.

Next, we investigated NSMCE2 recruitment in MEFs lacking FAN1 nuclease activity (*Fan1*$^{KI/KI}$, kind gift of Dr. John Rouse). First, biochemical fractionation experiments showed that the lack of nuclease activity did not affect the overall levels of chromatin-bound NSMCE2 (or SMC5) **(Figure 19e)**. Next, we investigated the dynamics of NSMCE2 recruitment to ICLs using psoralen-induced laser stripes in wild-type and FAN1 nuclease deficient MEF. Surprisingly, and in contrast to our observations in MMC-induced foci, NSMCE2 accumulates more intensely at psoralen-induced laser stripes in *Fan1*$^{KI/KI}$ MEFs **(Figure 19f)**. However, this is due to the higher load of damage that is induced by the treatment in the mutant MEFs, as reflected by a higher amount of H2AX phosphorylation at the laser stripes. In any case, both datasets converge in that NSMCE2 recruitment is not dependent on FAN1 or its nuclease activity.

In regards to FAN1 recruitment, the severe proliferation deficiency of *Nsmce2*-deleted cells while precluded a proper analysis of this by foci experiments, nevertheless chromatin fractionation experiments revealed that FAN1 recruitment to chromatin could still occur in the absence of NSMCE2 **(Figure 20)**. In summary, while FAN1 and NSMCE2 seem to recognize similar insults, they can do it independently.

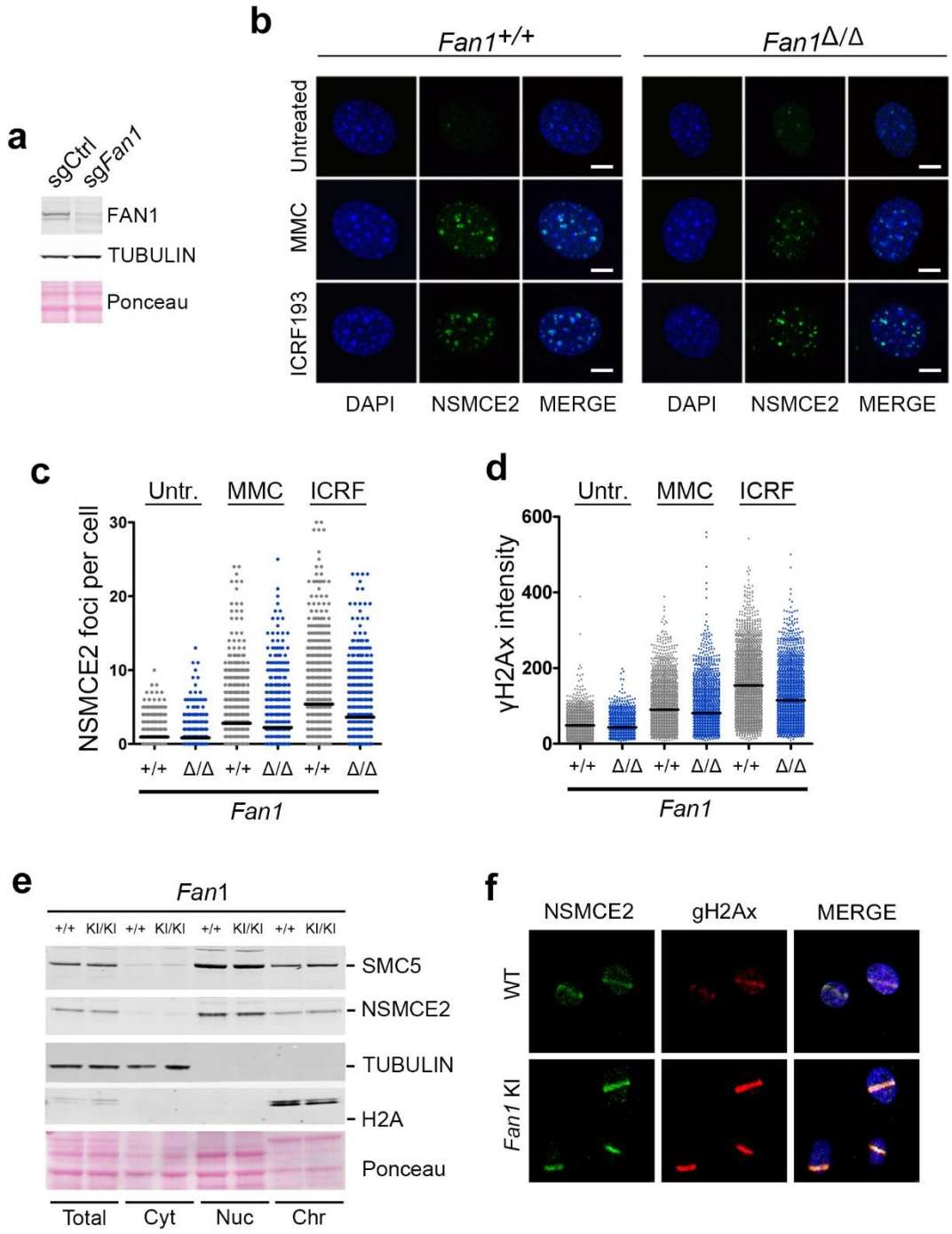

Fig. 19 - NSMCE2 recruitment is not dependent on FAN1.

a) WB illustrating the deletion of *Fan1* by CRISPR-Cas9 technology in MEFs. Control cells were infected with control sgRNA and 3 different sgRNAs were used to target the *Fan1* allele. Tubulin used as a loading control.

b) NSMCE2 foci in wild-type and *Fan1* knockout MEFs, treated with 300 nM MMC for 6 hours and 5µM ICRF-193 for 6 hours. Scale bar indicates 10 µm.

c) Quantification of foci mentioned in (b)

d) Analysis of γH2AX intensity by HTM in wild-type and *Fan1* knockout MEFs, treated with 300 nM MMC for 6 hours and 5 µM ICRF-193 for 6 hours.

e) NSMCE2 and SMC5 levels in total, cytosolic, soluble nuclear and chromatin fractions in wild-type and *Fan1*$^{KI/KI}$ MEFs.

f) NSMCE2 recruitment to psoralen-induced laser stripes in *Fan1*$^{KI/KI}$ MEFs. γH2AX was used to mark the sites of damage.

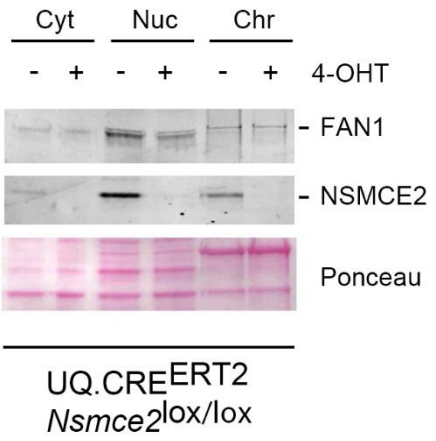

Fig. 20 - FAN1 localization is not affected by NSMCE2 loss.

Cellular fractionation of UQ. Cre^{ERT2} *Nsmce2*$^{lox/lox}$ treated for 48 hours with 4-OHT to induce *Nsmce2* deletion. Levels of chromatin-bound FAN1 do not vary in these cells.

2.9 - NSMCE2 and FAN1 are not epistatic

To gain further insights into the potential relationship between FAN1 and NSMCE2, double mutant cells were generated by using CRISPR-Cas9 technology to target *Fan1* in *Nsmce2* conditional knockout MEFs **(Figure 21a)**. These experiments revealed that the deletion of both *Nsmce2* and *Fan1* did not significantly increase the presence of segregation problems such as micronuclei or DNA bridges **(Figure 21c)**. These experiments confirmed the segregation problems induced by TOPOII inhibition of NSMCE2-deficient cells. In contrast, we did not observe any obvious impact of FAN1 deficiency in response to TOPOII inhibition, and the double mutant was as compromised as the single *Nsmce2* mutant. While we cannot discard that FAN1 might play some role in the response to topological stress under other conditions, these experiments show that the function of NSMCE2 in this response is independent of FAN1.

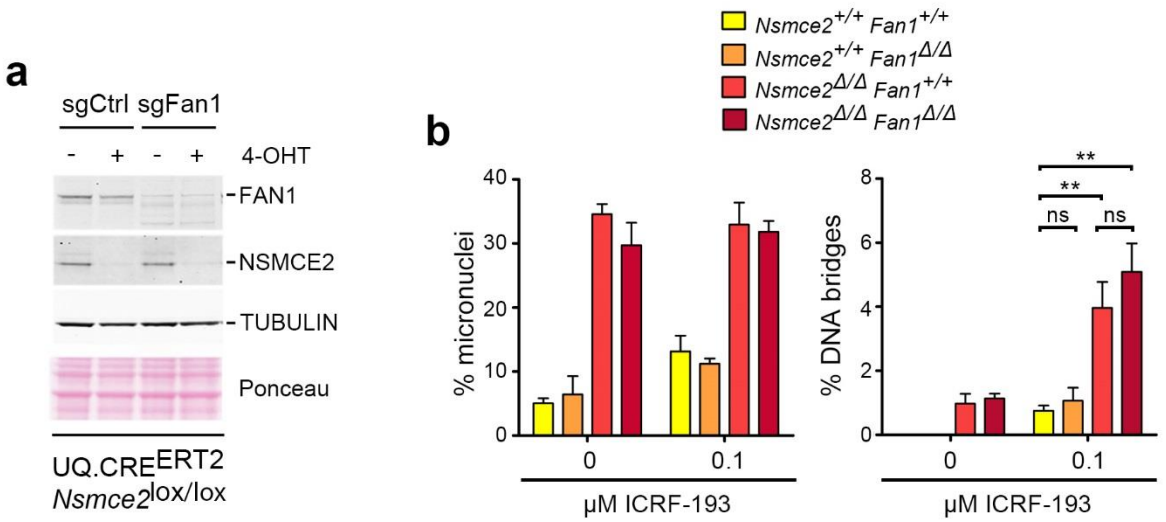

Fig. 21 - NSMCE2 and FAN1 are not epistatic.

a) WB illustrating the generation of *Fan1-Nsmce2* double mutant MEFs. UQ. Cre^{ERT2} *Nsmce2*$^{lox/lox}$ were infected with control sgRNA (lane 1) and treatment with 4-OHT of these cells generated *Nsmce2*$^{\Delta/\Delta}$ (lane2). 3 different sgRNAs were used to target *Fan1* allele in UQ. Cre^{ERT2} *Nsmce2*$^{lox/lox}$ (lane 3). Treatment with 4-OHT of *Fan1* knockout MEFs generated *Nsmce2-Fan1* double knockout cells. Tubulin used as a loading control.

b) Analysis of segregation defects (DNA bridges and micronuclei) induced by 16 hours ICRF-193 treatment in cells described in (a).

DISCUSSION

Discussion

Using mouse as a model system, our work shows that NSMCE2 is essential for embryonic development and that its post-natal deletion leads to accelerating ageing independently of its SUMO ligase activity. At the cellular level, the essential role of NSMCE2 resides in facilitating chromosome segregation likely through eliminating joint DNA molecules before they reach mitosis. In agreement with this view, NSMCE2 deficiency leads to a significant increase in Sister Chromatid Exchange (SCE) events, which arise through the processing of joint DNA molecules by structure-specific nucleases (Matos and West, 2014, West *et al*., 2015, Wyatt *et al*., 2013). Our current work supports that one key source of joint DNA molecules that is dealt by NSMCE2 (and, thus, by the SMC5/6 complex) are topological intertwines. Accordingly, NSMCE2 deficiency increases the levels of chromatin bound TOPOII, phenocopies several outcomes that arise upon TOPOII inhibition, and sensitizes cells to the effects of TOPOII inhibitors. *In vivo*, NSMCE2 deficiency leads to KIN, a kidney disorder that in humans was associated to exposure to OTA (TOPOII inhibitor) (Godin *et al*., 1996, Hassen *et al*., 2004, Cosimi *et al*., 2009), and to *FAN1* mutations (Zhou *et al*., 2012, Thongthip *et al*., 2016, Lachaud *et al*., 2016). Our work supports that the main function of the SMC5/6 complex is in the response to topological stress and that compromised topoisomerase function, rather than deficiencies in ICL-repair as previously thought, are the cause of KIN.

1 - Impact of *Nsmce2* deletion on mouse physiology

1.1 - The SMC5/6 complex suppresses cancer and ageing in mammals

Whereas the SMC5/6 complex has been subject of intensive research in yeast, its role in mammals has been less investigated. Previous to our work, two mouse models with mutations in SMC6 were described. Mice carrying an exon trap in the *Smc6* allele that abrogates the expression of the full-length SMC6 were generated by Ju *et al.* (2013). In agreement with our findings, loss of SMC6 was also embryonic lethal in mice. The same study reported that a mutation compromising the ATPase activity of SMC6 has in contrast no obvious impact in mouse physiology.

The lethality of *Nsmce2* deletion does not originate from the destabilization of the SMC5/6 complex, since we have also found that the complex is still able to form even in the absence of NSMCE2 (Jacome *et al*., 2015). Also, NSMCE2 deficiency does not affect the overall levels of chromatin bound SMC5/6 proteins. It is still possible that NSMCE2 might regulate

the recruitment of the SMC5/6 complex at particular regions. In this regard, an in-depth analysis of how *Nsmce2* deletion affects the genomewide distribution of SMC5/6 may provide insights in the understanding of how the SMC5/6 function is impaired. Alternatively, while NSMCE2 might be dispensable for the recruitment of SMC5/6 to its substrates, it might be important for its function. These are all still unresolved questions and while we know that the SUMO ligase activity is not the main determinant of the functions of NSMCE2, we do not know what or how NSMCE2 does to promote the functionality of the SMC5/6 complex.

In vivo, our work revealed that *Nsmce2* deletion in adult mice leads to accelerating ageing. In contrast, *Nsmce2* heterozygous mice are more prone to develop various types of cancer (Jacome *et al*., 2015) **(Figure 1)**. It so seems that while minor deficiencies in the complex can lead to malignancies, more severe mutations can lead to more severe physiological manifestations including accelerated ageing. The observation that the transition between cancer and aging depends on the severity of the mutation has been also reported for other pathways involved in genomic maintenance, including replication stress and the NER pathway (López-Contreras and Fernandez-Capetillo, 2010, Diderich *et al*., 2011).

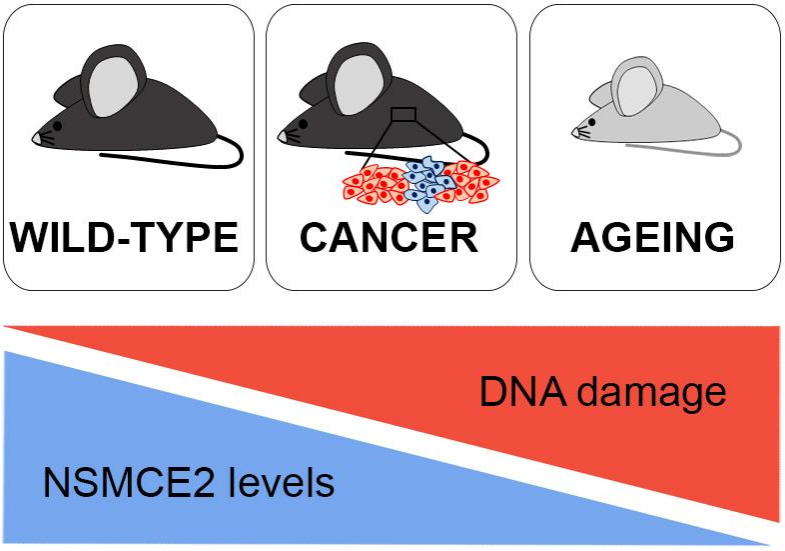

Fig. 1 - Impact of NSMCE2 levels on mouse physiology.

The transition between cancer and aging depends on the severity of the mutation. Whereas depletion of NSMCE2 in mice leads to accelerating ageing, reduced levels of the protein promote cancer development. Loss of NSMCE2 abrogates completely the proliferative ability of cells that undergo senescence. Residual activity of NSMCE2, although allowing proliferation, cannot promote an efficient repair of DNA damage. Hence, reduced NSMCE2 levels leads to increased chromosomal rearrangement and thus to cancer development.

We propose that the increased recombination rates linked to reduced NSMCE2 levels could be the source of cancer-initiating chromosomal rearrangements. In support of this, Mms21 and Smc6 were found as key factors in suppressing gross chromosomal rearrangements in *S. cerevisiae* (Albuquerque *et al*., 2013, De Piccoli *et al*., 2006). In addition, the SMC5/6 complex itself has been found in regions that are frequently rearranged in human leukaemias (Barlow *et al*., 2013), and fusions occurring at the *NSMCE2* locus have also been described in acute myeloid leukaemia (Chinen *et al*., 2014). To what extent these fusions and rearrangements compromise the function of the SMC5/6 complex and play a role in carcinogenesis is still not known.

In what regards to ageing, the most likely explanation is that this is due the major chromosome segregation problems that arise upon *Nsmce2* deletion, which will lead to the elimination of many of the cells in the adult mice. As observed in other mouse models of cell essential genes such as *Atr* (Ruzankina *et al*., 2007), the death of the mutant cells leads to a compensatory proliferation of the remaining wild-type cells, finally leading to the exhaustion of the regenerative capacities of the stem cells and ultimately ageing **(Figure 2)**. In addition to this, it is also possible that the accumulation of abnormal cells also plays a causal role in ageing. Accordingly, our present study shows that NSMCE2 deficiency leads to a distinct accumulation of polyploidy cells in the kidneys. Such a cell-type specific pattern has also been described in other mutations of DNA repair genes that promote progeria. For example, one of the most-studied models of progeria, linked to ERCC1 deficiency, also shows a preferential accumulation of abnormal cells in the liver (McWhir *et al*., 1993, Weeda *et al*., 1997).

Besides ageing, NSMCE2 deficiency results in a series of phenotypes that are also found in patients with other genomic instability syndromes. For instance, NSMCE2 deficiency leads to a significant increase in micronuclei, reduced fat percentage, altered pigmentation and mild anemia, which are characteristic of Bloom's Syndrome (de Renty and Ellis, 2017, German *et al*., 1974). In addition, the bone marrow failure and sensitivity to crosslinking agents that occurs upon NSMCE2 loss are also hallmarks of Fanconi anemia. As mentioned above, the severity and diversity of these phenotypes are common in mutations that compromise genomic stability. Along these lines, two human patients presenting reduced levels of NSMCE2 were also recently described (Payne *et al*., 2014). Similarly to our mouse model, these patients presented dwarfism, altered pigmentation, and increased micronuclei. Although these individuals did not show evident hallmarks of accelerating ageing, one of them died at the age of 33 from a sudden cardiovascular event. Of note, the milder phenotype observed in these two patients is likely due to the nature of the deficiency.

Whereas in our case we are studying the effects of *Nsmce2* nullyzygosity, both patients retain small levels of *NSMCE2* expression.

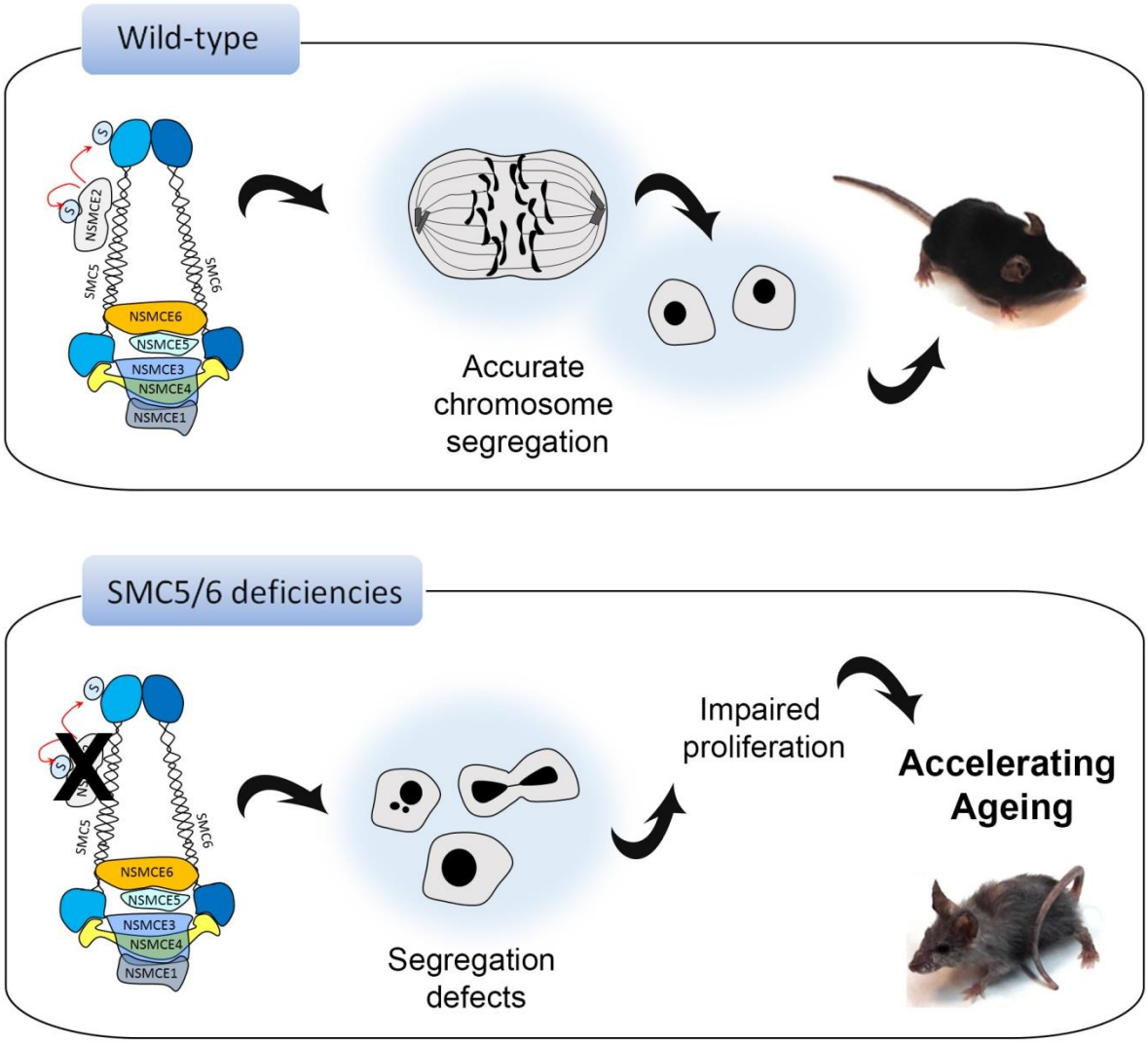

Fig. 2 - The SMC5/6 complex suppresses ageing by promoting accurate chromosome segregation.

Loss of NSMCE2 results in a series of segregation defects (micronuclei, DNA bridges and polyploidy) that impair cell growth, thus leading to accelerating cells in mice.

1.2 - The SUMO ligase activity of NSMCE2 is dispensable in mice

Our work illustrates that the essential functions of the SMC5/6 complex in mammals are independent of the SUMO ligase activity of NSMCE2. Indeed, NSMCE2 SUMOylation-deficient mice display a normal lifespan, whereas loss of the protein is embryonic lethal.

Likewise, no proliferation defects are displayed by cells deficient for NSMCE2 catalytic activity, while cells can only undergo limited rounds of cell divisions upon loss of NSMCE2. Similarly, the *Nse2* protein is essential for viability in yeasts, whereas its SUMO ligase activity is not (Andrews *et al*., 2005, McDonald *et al*., 2003, Xaver *et al*., 2013). One possible explanation, is that since SUMOylation is a rather redundant process the loss of NSMCE2-dependent SUMOylation could be compensated by alternative SUMO ligases. In support of this, the minor defects observed in SUMO-dead yeast strains are significantly aggravated when additional SUMO ligases are deleted (Albuquerque *et al*., 2013, Cremona *et al*., 2012). Moreover, although SUMOylation-deficient yeast strains are viable, they display hypersensitivity towards DNA damaging agents and aberrant chromosome segregation (Andrews *et al*., 2005, McDonald *et al*., 2003). Our observation on the dispensability of NSMCE2 SUMO ligase activity have been made in mice upon unchallenged conditions. However, we cannot discard that the SUMO ligase activity of NSMCE2 may become more relevant in the context of DNA damage. We should note, however, that SUMO ligase deficient mice are fertile, and meiosis involves a significant amount of DNA damage. Moreover, a recent report has shown that conditional deletion of SMC5 is essential for meiosis in oocytes (Hwang *et al*., 2017). Finally, and although the link between the SMC5/6 complex and SUMOylation appears independent of NSMCE2 activity, the complex may still retain some functions that are related to SUMO. For instance, it has been recently proposed that the SMC5/6 complex may act as a platform for SUMO, in order to bring SUMO where needed, such as sites of DNA damage (Bustard *et al*., 2016).

2 - The SMC5/6 complex suppresses recombination and facilitates chromosome segregation

The most evident cellular consequence of not having NSMCE2 is a remarkable growth impairment. For instance, while NSMCE2-deficient B-lymphocytes can undergo a few cell divisions *ex vivo*, they also present a high rate of cell death and the segregation problems leading to polyploidy. Also, MEFs become rapidly senescent after a few divisions upon *Nsmce2* deletion and also present major segregation problems. Of note, a recently developed *Smc5* conditional knockout allele has also observed major segregation problems in somatic and meiotic cells (Hwang *et al*., 2017). The ability to undergo some cell divisions in the absence of NSMCE2 suggests that problems build up throughout subsequent cell cycles until the damage is severe enough to trigger cell-death and/or senescence. Additionally, given that *Nsmce2* heterozygous tumours always retain the wild-type allele (Jacome *et al*., 2015) and that NSMCE*2* is also required for the proliferation of immortalized

MEFs, it seems likely that the SMC5/6 complex is also indispensable for the viability of cancer cells.

In absence of NSMCE2, overall replication occurs at a comparable rate to that of wild-type cells. We can, however, not rule out that replication at certain loci, such as at repeated regions, is affected by deficiencies in SMC5/6. The repetitive nature of these loci might lead to topological stress and/or DNA breakage later on depending on SMC5/6 for its resolution. In support of this, several studies demonstrated the localization of the different components of the SMC5/6 complex at repetitive sequences, including natural replication pausing sites, centromeres and telomeres (Ampatzidou *et al.,* 2006, Lindroos *et al*., 2006, Menolfi *et al*., 2015, Pebernard *et al*., 2008). Moreover, while restriction of the SMC5/6 complex expression to G2-M had no effect on overall origin firing and replication fork speed in yeast, it played a role on the replication of natural pausing sites (Menolfi *et al*., 2015).

Besides of any role of DNA replication, several studies have suggested that the complex participates in DSBs repair through homologous recombination (HR). However rather than participating in the actual repair process at all DNA breaks, as proposed by many of these works, we propose that the role of SMC5/6 in response to DSBs is linked to the generation of joint DNA molecules that might arise as by-products of the repair process at some DSBs. Thus, the role of SMC5/6 would be a later one, perhaps involved in the dissolution of entangled DNA molecules that reach mitosis, and that can arise during DNA repair. Consistently, expression of Smc6 in mitosis is sufficient for the removal of the recombination molecules previously accumulated in *Smc6* mutant yeast (Bermúdez-López *et al*., 2010). In any case, the fact that at least a subset of the joint DNA molecules that accumulate in SMC5/6 mutants depend on RAD51, argues that they are indeed generated during HR repair (Ampatzidou *et al*., 2006, Branzei *et al*., 2006, Bermúdez-López *et al*., 2010, Yong-Gonzales *et al*., 2012, Torres-Rosell *et al*., 2005).

Rather than seen a decrease in HR, and in agreement with previous reports (Prakash and Prakash, 1977, Kliszczak *et al*., 2012, Ju *et al*., 2013), our results show that NSMCE2 deficiency in mouse cells leads to increased recombination rates as detected by SCEs. We believe that this is secondary to the accumulation of joint DNA molecules. The elimination of these entanglements is mediated either by the dissolution pathway involving the BLM helicase, or through a nuclease-dependent pathway known as resolution (Mankouri *et al*., 2013, Matos and West, 2014). When dissolution fails, as in BLM deficient cells, the only pathway left is resolution leading to the breakage of joint DNA molecules and SCE events. This recombination phenotype can be reverted by the abrogation of MUS81 function, thus confirming that SCE is resolution-dependent event in these cells (Jacome *et al*., 2015, Xaver

et al., 2013). The MUS81-dependent increase in SCEs observed in NSMCE2-deficient cells would suggest that its role is analogous to that of BLMs.

Since mutations in the SMC5/6 complex resemble BLM deficiencies, it has been hypothesized that these factors could work together in order to promote HJ dissolution. In fact, NSMCE2 deficiency in mice recapitulates some of the characteristics of BLM patients. This similarity between SMC5/6 and BLM was previously noted in several yeast studies. For example, yeast mutants of *sgs1*, the orthologue of BLM, also accumulate recombination intermediates in a RAD51-dependent manner (Liberi *et al*., 2005, Bermúdez-López *et al*., 2016, Branzei *et al*., 2006, Bonner *et al*., 2016). Despite the resemblance of BLM and NSMCE2 phenotypes, early studies in yeasts failed to link the activity of the SMC5/6 complex to the one of Sgs1 (Branzei *et al*., 2006). However, more recently, a physical interaction between Smc5 and sgs1 was demonstrated in yeasts (Bonner *et al.*, 2016, Bermúdez-López *et al*., 2016). The purpose of this interaction resides in the SUMOylation of the three components of the BTR complex. The SUMOylation of the BTR complex is partially dependent on Mms21 activity and it is essential to counteract the accumulation of DNA joint molecules (Bermúdez-López *et al*., 2016, Bonner *et al*., 2016). Even if SUMOylation deficiency is not pathological in mice, this does not discard the possibility that BLM and SMC5/6 work on the resolution of a common problem. In agreement with this, the deletion of BLM in combination with NSMCE2 deficiency is synthetic lethal in mammalian cells (Jacome *et al*., 2015) and in yeast (Chen *et al*., 2009). In addition, double mutant yeasts accumulate further DNA joint molecules in mitosis (Torres-Rosell *et al*., 2005) and meiosis (Xaver *et al*., 2013, Copsey *et al*., 2013).

3 - NSMCE2 and TOPO2 work together to ensure chromosome segregation

Our data supports that one of the key functions held by the SMC5/6 complex involves the cooperation with TOPOII in dealing with topological problems arising from chromatin transactions. Due to the intrinsic nature of DNA, chromatin processes such as replication, transcription and repair can generate topological problems during the unwinding of the duplex DNA and the exposure of ssDNA. Topoisomerases are the best-established component in limiting topological stress. In particular, TOPOII works by the introduction of a transient DSB, followed by a DNA conformational change to eliminate the supercoil and the relegation of the ssDNA ends (Nitiss, 2009). Today, many lines of evidence support that the role of the SMC5/6 complex is linked to TOPOII.

TOPOII deficiency is embryonic lethal in yeast (Holm *et al*., 1985, Uemura and Yanagida, 1984, DiNardo *et al*., 1984) and mice (Akimitsu *et al*., 2003). Interestingly, embryos lacking TOPOII present the same mitotic abnormalities that we could observe in early NSMCE2-deficient embryos (Jacome *et al.*, 2015). Moreover, segregation deficiencies in NSMCE2-deficient MEFs seem to preferentially involve pericentromeric DNA, which is the largest repeat of the mammalian genome. Likewise, loss of TOPOII results in failure of centromeric disjunction, with an increased rate of segregation defects including DNA bridges and lagging chromosomes (Coelho *et al*., 2008, Bower *et al*., 2010). ChIP-seq analysis further supports the resemblance of SMC5/6 and TOPOII. These studies have revealed that SMC5/6 localizes preferentially associates with repeated regions in yeast like centromeres or ribosomal DNA (Lindroos *et al*., 2006, Jeppsson *et al*., 2014). In the case of TOPOII, it is well known that it is particularly relevant for the segregation of centromeric regions (Rattner *et al*., 1996, Christensen *et al*., 2002), and our immunofluorescence analyses support such a dominant role since TOPOII is particularly enriched around pericentromeric DNA. Moreover, upon TOPOII inhibition NSMCE2 rapidly relocalizes to heterochromatin, further supporting a coordinated role of these complexes in dealing with topological stress. Finally, the link to TOPOII is further suggested by the finding of diplochromosomes in the metaphases of *Nsmce2* knockout MEFs, an event that is also induced by TOPOII is inhibition (Sumner, 1998, Ji *et al*., 2009, Cortés and Pastor, 2003).

The many similarities of TOPOII and NSMCE2 deficiencies argue for a similar function of these factors, but how they work together, if they do, is still unclear. A model for TOPOII-SMC5/6 interaction was proposed by Kegel *et al.* (2011). According to this study, the SMC5/6 complex would reduce the superhelical tension ahead of the replication fork by promoting fork reversal **(Figure 3)**. Fork reversal would then generate sister chromatids interwinings (SCIs) which are known substrates for TOPOII. Therefore, the function of the SMC5/6 complex would reside in the generation of DNA substrates with the appropriate conformation for TOPOII resolution. This view is supported by the accumulation of SCIs in *top2-4* mutant yeasts, since TOPOII is necessary to dissolve these structures. Moreover, this increase in SCIs is reduced in *smc6-56 top2-4* double mutant, supporting the role of the SMC5/6 complex in fork reversal-mediated generation of SCIs (Kegel *et al*., 2011).

Several reports have demonstrated a genetic interaction between the SMC5/6 complex and TOPOII in yeasts (Takahashi *et al.*, 2008, Outwin *et al.*, 2009, Jeppsson *et al*., 2014). The synthetic lethality observed in these mutants suggests that both complexes might deal independently in the resolution of HJs. In support of this, and while TOPOII and NSMCE2 colocalize at foci, we failed to see a direct interaction between these complexes by IP. We shall note that a recent manuscript has reported a coIP between TOPOII and SMC5, but this

is something we cannot reproduce (Verver *et al*., 2016). In any case, both our work and this manuscript revealed an increased sensitivity of SMC5/6 mammalian mutants to TOPO inhibition, providing the first evidences of a synthetic lethal interaction of TOPOII and SMC5/6 in mammalian cells.

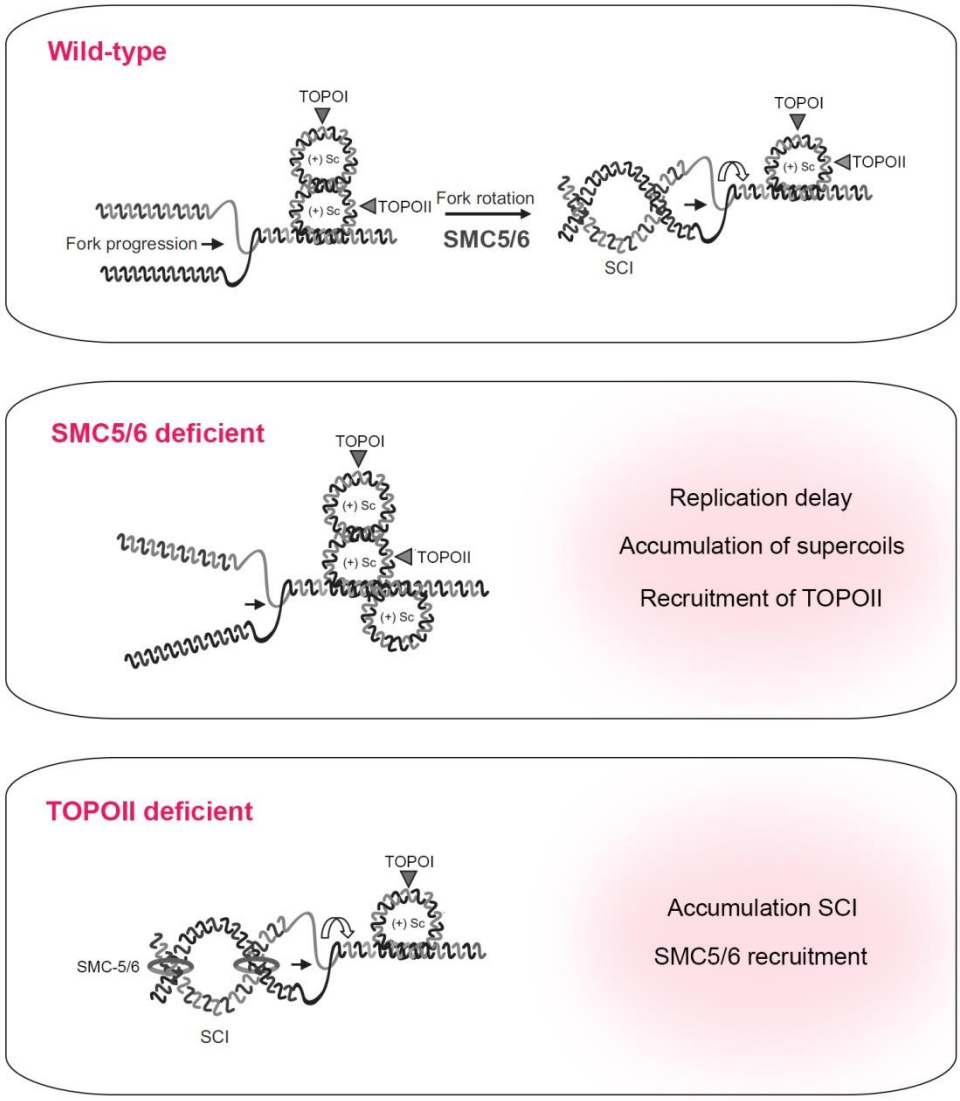

Fig. 3 - The SMC5/6 promotes fork reversal and generates TOPOII-competent substrates. Modified from Kegel and Sjogren (2010).

DNA replication generates positive supercoiled DNA ahead of the fork. Mainly Top1, but also Top2, alleviates this superhelical tension in order to allow fork progression. The SMC5/6 complex might promote fork reversal that generates SCIs. Top2 plays a central role in removing these catenates.

As regards the type of lesion recognised by the SMC5/6 complex, it seems clear recombination intermediates are not the only substrates and other will likely be sites of

topological stress. Accordingly, top2 deletion in yeast leads to a redistribution of Smc6 along the chromosome arms that is independent of recombination. Moreover, no recombination intermediates were identified in regions where SMC5/6 accumulates in the absence of TOPOII (Jeppsson *et al*., 2014). Instead of sites of recombination, and since deficiencies in TOPOII result in SCIs accumulation, SMC5/6 association to chromatin may be triggered by SCIs in these mutants **(Figure 3)**. In absence of TOPOII, the SMC5/6 complex may facilitate the resolution of SCIs, however not as efficiently as when TOPOII is present. The SMC5/6 complex might be able to recognise distinct structures that perturb chromatin architecture. SCIs and recombination intermediates must share some structural features able to trigger the association of the complex.

To end, we should end by noting that the similar phenotype of FAN1 and NSMCE2 deficiency in mice, namely the appearance of KIN, could suggest that FAN1 might participate in the response to topological stress. This is interesting since even if FAN1 was first associated to ICL-repair, *FAN1* mutant patients do not have Fanconi Anemia (Lans and Hoeijmakers, 2012, Zhou *et al*., 2012, Trujillo *et al*., 2012) and the only phenotype that they present is KIN. Moreover, the ICL-repair defect of FAN1-deficient cells is modest, raising doubts of what is the real substrate of this nuclease. The resemblance of the phenotypes could suggest that FAN1's true substrates are HJs or other sites of topological stress. While in an initial experiment we failed to observed a distinctively high toxicity of TOPOII inhibitors in FAN1-deficient cells, this line of work deserves further exploration in depth (see below).

4 - Karyomegalic interstitial nephritis as a result of topological problems

Here we report the second mutation, after *FAN1* that can lead to KIN in mammals. Once again, the role of NSMCE2 in KIN is independent of its SUMO ligase activity, further illustrating that NSMCE2 plays important functions in SMC5/6 besides its SUMO ligase activity. Our data, together with evidence provided by other studies, suggest that KIN is a consequence of the accumulation of topological stress that ultimately leads to polyploidy **(Figure 4)**. Our view that KIN is linked to topological stress is supported by the fact that in human patients, KIN was found to be primarily associated to the contact with the mycotoxin OTA (Hassen *et al*., 2004, Godin *et al*., 1996), which was later found to inhibit TOPOII (Cosimi *et al*., 2009). Furthermore, TOPOII expression is distinctively high in karyomegalic cells found in the renal tubuli (Adler *et al.*, 2009). Similarly, NSMCE2 deficiency in MEFs produces a pronounced accumulation of TOPOII at centromeres. Moreover, many reports have shown a genetic relationship between the SMC5/6 complex and TOPOII (Jeppsson *et al*., 2014, Kegel *et al*., 2011, Takahashi *et al.*, 2008, Outwin *et al*., 2009).

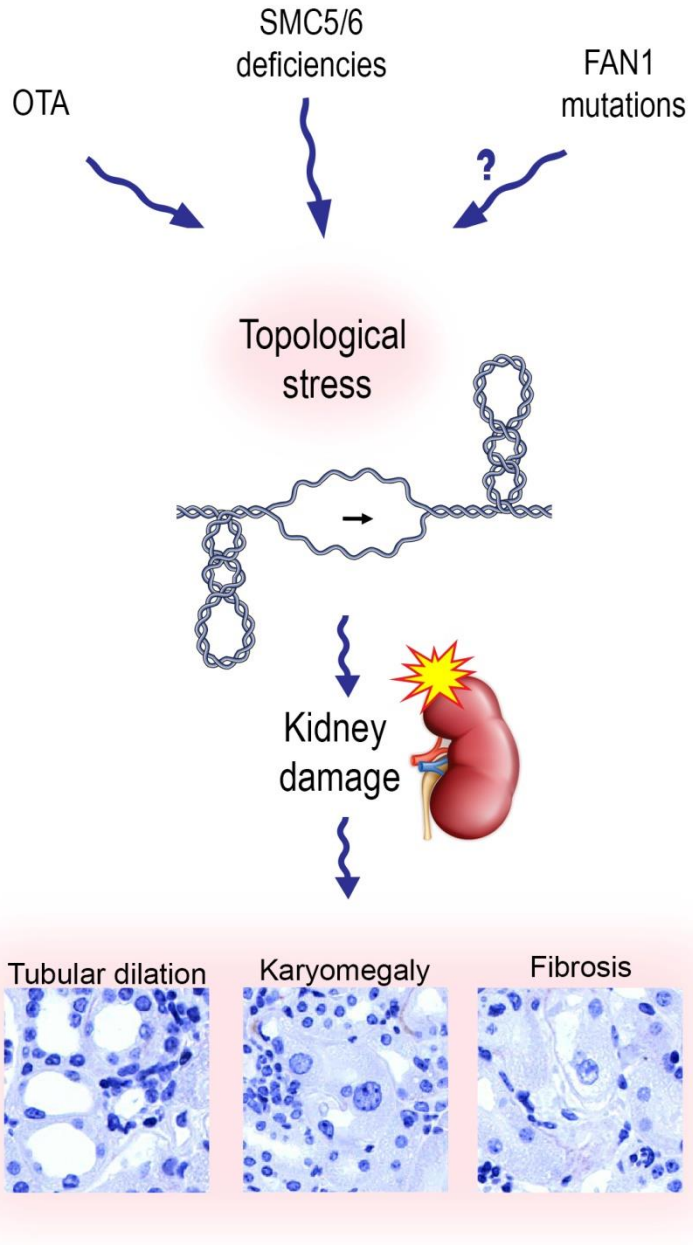

Fig. 4 - Topological stress can trigger the onset of KIN.

OTA and NSMCE2 deficiency induce KIN by altering the activity of TOPOII. Whereas OTA is able inhibit topoisomerase activity *in vitro,* NSMCE2 loss triggers a significant association of TOPOII to centromeres. The link with topological stress is still unclear for mutations in the nuclease *FAN1,* also associated to KIN phenotype in mammals.

As mentioned, mutations in the *FAN1* nuclease are the only known cause of hereditary KIN in humans (Zhou *et al*., 2012, Lachaud *et al*., 2016, Thongthip *et al*., 2016). Since early studies linked the activity of FAN1 to the FA pathway (Smogorzewska et al., 2010, MacKay

et al., 2010), it was proposed that FAN1 could participate in the repair of ICLs and that the KIN phenotype would arise from the defective repair of these DNA lesions. However, the clinical outcome of FAN1 deficiencies and FA differs significantly. Individuals carrying mutations in *FAN1* are negative for FA testing and they do not develop anemia (Lans and Hoeijmakers, 2012, Zhou *et al*., 2012, Trujillo *et al*., 2012). Moreover, no evidences of KIN have been found in the kidneys from FA patients and FA mouse models. The evaluation of the epistatic relation of FAN1 with the FA proteins FANCD2 and SLX4 have further suggested a minor role for FAN1 in the ICL repair pathway (Thongthip *et al*., 2016, Lachaud *et al*., 2016). These observations suggest that KIN development is not strictly connected to deficiencies in ICL repair, but other mechanism, for instance topological stress, could trigger the disease.

The fact that catalytic inhibitors of TOPOII promote robust FAN1 foci shows, for the first time, that FAN1 plays a role in the response to topological problems. In fact, the inhibition of TOPOII generates a more severe lesion that recruits FAN1 in a greater number of cells than ICL-inducing agents. Whereas in a first attempt we failed to detect an obvious sensitivity of FAN1-deficient cells to TOPOII inhibition, this needs to be further addressed in other cell types and experimental conditions. It is also possible that the role of NSMCE2, and thus of SMC5/6 is more critical than that of FAN1. In fact, the KIN that occurs upon *Fan1* deletion in mice is significantly milder than what we see in NSMCE2-deficient mice (Thongthip *et al*., 2016). While KIN is only fully developed after 1 year in FAN1-deficient mice, we can see severely affected kidneys in mice that are five months of age in *Nsmce2* mutants. It would be interesting to see whether a treatment with OTA or ICRF-193 further accelerates the onset of KIN in either of the strains.

Beside renal disorders, FAN1 deficiency in mice has been also associated to karyomegaly and dysfunction in liver (Thongthip *et al*., 2016). In contrast, we have not identified an obvious phenotype in NSMCE2-deficient livers. Why some organs are more affected by a mutation than by another one remains enigmatic. It is not surprising however that kidney and liver are the most affected organs. These organs are continuously exposed to genotoxic insults coming from waste metabolites. For this reason, liver and kidney naturally endure higher doses of DNA damage compared to other organs. One possibility is that these organs have selected a higher threshold for triggering apoptosis in response to DNA damage, to prevent excessive cell elimination due to the contact with toxins. In support of this, it is noteworthy that the karyomegaly in KIN is restricted to proximal tubular epithelial cells that are in a more direct contact with toxic metabolites, whereas the renal medulla remains almost unaffected.

In regards to the nature of the KIN-initiating insult, environmental exposure to toxins, such as OTA (Hassen *et al*., 2004, Godin *et al.*, 1996) or various chemotherapeutics (McCulloch *et al*., 2011, Mihatsch *et al.*, 1979, Burry, 1974, Godin *et al*., 1996) can damage kidneys and lead to renal failure. Interestingly, kidneys can also suffer from the products of our own endogenous metabolism. Given the increasing evidence that metabolism-generated formaldehyde and acetaldehyde able to induce cellular damage, it is possible that aldehydes can contribute to KIN pathogenesis. The sensitivity to acetaldehyde and formaldehyde of FAN1-deficient cells favour this association (Thongthip *et al*., 2016). The sensitivity towards these compounds upon *Nsmce2* deletion would provide further evidence of the link between kidney disorders and endogenous genotoxic metabolites. To explore this possibility, in early studies of my PhD I attempted to rescue the viability of NSMCE2-deficient MEFs by overexpressing ADH5, the main enzyme in formaldehyde catabolism. However, this experiment failed, although acknowledgedly we did not pursue this in sufficient depth. A genetically cleaner way could be to test if KIN is aggravated ADH5-NSMCE2 double mutant mice. If true, this would suggest that the initial insult that triggers KIN, even if this is later converted into topological stress, is the presence of formaldehyde. Along these lines, a recent study showed the ability of formaldehyde to induce DNA damage in kidneys (Pontel *et al*., 2015). Moreover, and while FANCD2-deficient mice do not suffer from kidney disorders, a disease with resemblances to KIN does occur when FANCD2 deficiency occurs in combination with ADH5 deletion. Whereas we favour that the role of FAN1 might be more linked to topological stress than to ICL-repair, one interesting possibility is that FAN1 could be directly involved in the repair of the crosslinks induced by the formaldehyde, whereas the role of SMC5/6 could be more associated to HJs that could arise secondarily at ICL sites.

CONCLUSIONS

Conclusions

- NSMCE2 deficiency is dispensable for overall DNA replication but cell essential due to chromosome segregation defects.
- NSMCE2 suppresses cancer and ageing in mice independently of its SUMO-ligase activity.
- *Nsmce2* deletion in adult mice leads to Karyomegalic Interstitial Nephritis (KIN), which is characterized by the presence of karyomegalic cells in the cortex of the kidneys, ultimately leading to tubular dilation, fibrosis and compromised kidney function.
- The onset of KIN is independent of the SUMO ligase activity of NSMCE2.
- In addition to its recruitment to sites of DNA damage or interstrand-crosslinks, NSMCE2 forms prominent nuclear foci in response to TOPOII inhibition.
- NSMCE2 deficiency increases the levels of chromatin-bound TOPOII.
- NSMCE2 deficiency phenocopies TOPOII inhibition in various aspects such as the appearance of diplochromosomes or anaphase bridges involving pericentromeric regions.
- NSMCE2-deficient cells are sensitive to the segregation defects induced by TOPOII inhibition.
- While FAN1 colocalizes with NSMCE2 at sites of topological stress, *Fan1* deletion does not sensitize MEFs to TOPOII inhibition.

CONCLUSIONES

Conclusiones

- NSMCE2 no es indispensable para el proceso de replicación del DNA, sin embargo resulta esencial para asegurar una correcta segregación cromosómica.
- NSMCE2 suprime el cáncer y el envejecimiento en ratones independientemente de su actividad como SUMO ligasa.
- La deleción de *Nsmce2* en animales adultos produce nefritis intersticial cariomegálica, que se caracteriza por la presencia de células cariomegálicas en la corteza renal. En último término da lugar a dilatación tubular, fibrosis, y compromete la función renal.
- La aparición de nefritis intersticial cariomegálica es independiente de la actividad SUMO ligasa de NSMCE2.
- Además de acumularse en áreas dañadas del DNA o “interstrand crosslinks”, NSMCE2 forma focos nucleares en respuesta a inhibidores de la TOPOII.
- La deficiencia en NSMCE2 incrementa los niveles de TOPOII unida a cromatina.
- La deficiencia en NSMCE2 fenocopia la inhibición de TOPOII en varios aspectos, como la aparición de diplocromosomas o puentes de cromatina en regiones pericentroméricas.
- Las células deficientes en NSMCE2 son sensibles a los defectos en segregación cromosómica inducidos por la inhibición de TOPOII.
- Mientras FAN1 colocaliza con NSMCE2 en regiones de stress topológico, la deleción de *Fan1* no conduce a la sensibilización de fibroblastos (MEFs) frente a la inhibición de TOPOII.

BIBLIOGRAPHY

Bibliography

Adler, M., Müller, K., Rached, E., Dekant, W. and Mally, A. (2009) 'Modulation of key regulators of mitosis linked to chromosomal instability is an early event in ochratoxin A carcinogenicity', *Carcinogenesis,* 30(4), pp. 711-9.

Akimitsu, N., Adachi, N., Hirai, H., Hossain, M. S., Hamamoto, H., Kobayashi, M., Aratani, Y., Koyama, H. and Sekimizu, K. (2003) 'Enforced cytokinesis without complete nuclear division in embryonic cells depleting the activity of DNA topoisomerase IIalpha', *Genes Cells,* 8(4), pp. 393-402.

Albuquerque, C. P., Wang, G., Lee, N. S., Kolodner, R. D., Putnam, C. D. and Zhou, H. (2013) 'Distinct SUMO ligases cooperate with Esc2 and Slx5 to suppress duplication-mediated genome rearrangements', *PLoS Genet,* 9(8), pp. e1003670.

Aguilera, A. and Gómez-González, B. (2008) 'Genome instability: a mechanistic view of its causes and consequences', *Nat Rev Genet,* 9(3), pp. 204-17.

Ampatzidou, E., Irmisch, A., O'Connell, M. J. and Murray, J. M. (2006) 'Smc5/6 is required for repair at collapsed replication forks', *Mol Cell Biol,* 26(24), pp. 9387-401.

Andrews, E. A., Palecek, J., Sergeant, J., Taylor, E., Lehmann, A. R. and Watts, F. Z. (2005) 'Nse2, a component of the Smc5-6 complex, is a SUMO ligase required for the response to DNA damage', *Mol Cell Biol,* 25(1), pp. 185-96.

Barlow, J. H., Faryabi, R. B., Callén, E., Wong, N., Malhowski, A., Chen, H. T., Gutierrez-Cruz, G., Sun, H. W., McKinnon, P., Wright, G., Casellas, R., Robbiani, D. F., Staudt, L., Fernandez-Capetillo, O. and Nussenzweig, A. (2013) 'Identification of early replicating fragile sites that contribute to genome instability', *Cell,* 152(3), pp. 620-32.

Bartek, J., Bartkova, J. and Lukas, J. (2007) 'DNA damage signalling guards against activated oncogenes and tumour progression', *Oncogene,* 26(56), pp. 7773-9.

Benbow, R. M., Zuccarelli, A. J. and Sinsheimer, R. L. (1975) 'Recombinant DNA molecules of bacteriophage phi chi174', *Proc Natl Acad Sci U S A,* 72(1), pp. 235-9.

Bermúdez-López, M., Ceschia, A., de Piccoli, G., Colomina, N., Pasero, P., Aragón, L. and Torres-Rosell, J. (2010) 'The Smc5/6 complex is required for dissolution of DNA-mediated sister chromatid linkages', *Nucleic Acids Res,* 38(19), pp. 6502-12.

Bermúdez-López, M., Villoria, M. T., Esteras, M., Jarmuz, A., Torres-Rosell, J., Clemente-Blanco, A. and Aragon, L. (2016) 'Sgs1's roles in DNA end resection, HJ dissolution, and crossover suppression require a two-step SUMO regulation dependent on Smc5/6', *Genes Dev,* 30(11), pp. 1339-56.

Bonner, J. N., Choi, K., Xue, X., Torres, N. P., Szakal, B., Wei, L., Wan, B., Arter, M., Matos, J., Sung, P., Brown, G. W., Branzei, D. and Zhao, X. (2016) 'Smc5/6 Mediated Sumoylation

of the Sgs1-Top3-Rmi1 Complex Promotes Removal of Recombination Intermediates', *Cell Rep,* 16(2), pp. 368-78.

Bower, J. J., Karaca, G. F., Zhou, Y., Simpson, D. A., Cordeiro-Stone, M. and Kaufmann, W. K. (2010) 'Topoisomerase IIalpha maintains genomic stability through decatenation G(2) checkpoint signaling', *Oncogene,* 29(34), pp. 4787-99.

Branzei, D., Sollier, J., Liberi, G., Zhao, X., Maeda, D., Seki, M., Enomoto, T., Ohta, K. and Foiani, M. (2006) 'Ubc9- and mms21-mediated sumoylation counteracts recombinogenic events at damaged replication forks', *Cell,* 127(3), pp. 509-22.

Burry, A. F. (1974) 'Extreme dysplasia in renal epithelium of a young woman dying from hepatocarcinoma', *J Pathol,* 113(3), pp. 147-50.

Bustard, D. E., Ball, L. G. and Cobb, J. A. (2016) 'Non-Smc element 5 (Nse5) of the Smc5/6 complex interacts with SUMO pathway components', *Biol Open,* 5(6), pp. 777-85.

Bustard, D. E., Menolfi, D., Jeppsson, K., Ball, L. G., Dewey, S. C., Shirahige, K., Sjögren, C., Branzei, D. and Cobb, J. A. (2012) 'During replication stress, non-SMC element 5 (NSE5) is required for Smc5/6 protein complex functionality at stalled forks', *J Biol Chem,* 287(14), pp. 11374-83.

Campisi, J. (2003) 'Cancer and ageing: rival demons?', *Nat Rev Cancer,* 3(5), pp. 339-49.

Carrero, D., Soria-Valles, C. and López-Otín, C. (2016) 'Hallmarks of progeroid syndromes: lessons from mice and reprogrammed cells', *Dis Model Mech,* 9(7), pp. 719-35.

Ceccaldi, R., Sarangi, P. and D'Andrea, A. D. (2016) 'The Fanconi anaemia pathway: new players and new functions', *Nat Rev Mol Cell Biol,* 17(6), pp. 337-49.

Cejka, P., Plank, J. L., Bachrati, C. Z., Hickson, I. D. and Kowalczykowski, S. C. (2010) 'Rmi1 stimulates decatenation of double Holliday junctions during dissolution by Sgs1-Top3', *Nat Struct Mol Biol,* 17(11), pp. 1377-82.

Chan, Y. W. and West, S. (2015) 'GEN1 promotes Holliday junction resolution by a coordinated nick and counter-nick mechanism', *Nucleic Acids Res,* 43(22), pp. 10882-92.

Chan, Y. W. and West, S. C. (2014) 'Spatial control of the GEN1 Holliday junction resolvase ensures genome stability', *Nat Commun,* 5, pp. 4844.

Chen, Y. H., Choi, K., Szakal, B., Arenz, J., Duan, X., Ye, H., Branzei, D. and Zhao, X. (2009) 'Interplay between the Smc5/6 complex and the Mph1 helicase in recombinational repair', *Proc Natl Acad Sci U S A,* 106(50), pp. 21252-7.

Chinen, Y., Sakamoto, N., Nagoshi, H., Taki, T., Maegawa, S., Tatekawa, S., Tsukamoto, T., Mizutani, S., Shimura, Y., Yamamoto-Sugitani, M., Kobayashi, T., Matsumoto, Y., Horiike, S., Kuroda, J. and Taniwaki, M. (2014) '8q24 amplified segments involve novel fusion genes between NSMCE2 and long noncoding RNAs in acute myelogenous leukemia', *J Hematol Oncol,* 7, pp. 68.

Christensen, M. O., Larsen, M. K., Barthelmes, H. U., Hock, R., Andersen, C. L., Kjeldsen, E., Knudsen, B. R., Westergaard, O., Boege, F. and Mielke, C. (2002) 'Dynamics of human DNA topoisomerases IIalpha and IIbeta in living cells', *J Cell Biol,* 157(1), pp. 31-44.

Ciccia, A. and Elledge, S. J. (2010) 'The DNA damage response: making it safe to play with knives', *Mol Cell,* 40(2), pp. 179-204.

Coelho, P. A., Queiroz-Machado, J., Carmo, A. M., Moutinho-Pereira, S., Maiato, H. and Sunkel, C. E. (2008) 'Dual role of topoisomerase II in centromere resolution and aurora B activity', *PLoS Biol,* 6(8), pp. e207.

Collado, M., Blasco, M. A. and Serrano, M. (2007) 'Cellular senescence in cancer and aging', *Cell,* 130(2), pp. 223-33.

Copsey, A., Tang, S., Jordan, P. W., Blitzblau, H. G., Newcombe, S., Chan, A. C., Newnham, L., Li, Z., Gray, S., Herbert, A. D., Arumugam, P., Hochwagen, A., Hunter, N. and Hoffmann, E. (2013) 'Smc5/6 coordinates formation and resolution of joint molecules with chromosome morphology to ensure meiotic divisions', *PLoS Genet,* 9(12), pp. e1004071.

Cortés, F. and Pastor, N. (2003) 'Induction of endoreduplication by topoisomerase II catalytic inhibitors', *Mutagenesis,* 18(2), pp. 105-12.

Cosimi, S., Orta, L., Mateos, S. and Cortés, F. (2009) 'The mycotoxin ochratoxin A inhibits DNA topoisomerase II and induces polyploidy in cultured CHO cells', *Toxicol In Vitro,* 23(6), pp. 1110-5.

Cremona, C. A., Sarangi, P., Yang, Y., Hang, L. E., Rahman, S. and Zhao, X. (2012) 'Extensive DNA damage-induced sumoylation contributes to replication and repair and acts in addition to the mec1 checkpoint', *Mol Cell,* 45(3), pp. 422-32.

D'Andrea, A. D. and Grompe, M. (2003) 'The Fanconi anaemia/BRCA pathway', *Nat Rev Cancer,* 3(1), pp. 23-34.

De Piccoli, G., Cortes-Ledesma, F., Ira, G., Torres-Rosell, J., Uhle, S., Farmer, S., Hwang, J. Y., Machin, F., Ceschia, A., McAleenan, A., Cordon-Preciado, V., Clemente-Blanco, A., Vilella-Mitjana, F., Ullal, P., Jarmuz, A., Leitao, B., Bressan, D., Dotiwala, F., Papusha, A., Zhao, X., Myung, K., Haber, J. E., Aguilera, A. and Aragón, L. (2006) 'Smc5-Smc6 mediate DNA double-strand-break repair by promoting sister-chromatid recombination', *Nat Cell Biol,* 8(9), pp. 1032-4.

de Renty, C. and Ellis, N. A. (2017) 'Bloom's syndrome: Why not premature aging?: A comparison of the BLM and WRN helicases', *Ageing Res Rev,* 33, pp. 36-51.

Deans, A. J. and West, S. C. (2011) 'DNA interstrand crosslink repair and cancer', *Nat Rev Cancer,* 11(7), pp. 467-80.

Diderich, K., Alanazi, M. and Hoeijmakers, J. H. (2011) 'Premature aging and cancer in nucleotide excision repair-disorders', *DNA Repair (Amst),* 10(7), pp. 772-80.

DiNardo, S., Voelkel, K. and Sternglanz, R. (1984) 'DNA topoisomerase II mutant of Saccharomyces cerevisiae: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication', *Proc Natl Acad Sci U S A,* 81(9), pp. 2616-20.

Doniger, J., Warner, R. C. and Tessma, I. (1973) 'Role of circular dimer DNA in the primary recombination mechanism of bacteriophage S13', *Nat New Biol,* 242(114), pp. 9-12.

Duan, X., Yang, Y., Chen, Y. H., Arenz, J., Rangi, G. K., Zhao, X. and Ye, H. (2009) 'Architecture of the Smc5/6 Complex of Saccharomyces cerevisiae Reveals a Unique Interaction between the Nse5-6 Subcomplex and the Hinge Regions of Smc5 and Smc6', *J Biol Chem,* 284(13), pp. 8507-15.

Fanconi, G., Familiäre infantile perniziosaartige Anämie (perniziöses Blutbild und Konstitution). Jahrbuch für Kinderheilkunde und physische Erziehung (Wien), 11 7, pp. 257–280 (1927) (in German).

Fernandez-Capetillo, O. (2016) 'The (elusive) role of the SMC5/6 complex', *Cell Cycle,* 15(6), pp. 775-6.

Finkel, T., Serrano, M. and Blasco, M. A. (2007) 'The common biology of cancer and ageing', *Nature,* 448(7155), pp. 767-74.

Forget, A. L. and Kowalczykowski, S. C. (2010) 'Single-molecule imaging brings Rad51 nucleoprotein filaments into focus', *Trends Cell Biol,* 20(5), pp. 269-76.

Friedrich, K., Lee, L., Leistritz, D. F., Nürnberg, G., Saha, B., Hisama, F. M., Eyman, D. K., Lessel, D., Nürnberg, P., Li, C., Garcia-F-Villalta, M. J., Kets, C. M., Schmidtke, J., Cruz, V. T., Van den Akker, P. C., Boak, J., Peter, D., Compoginis, G., Cefle, K., Ozturk, S., López, N., Wessel, T., Poot, M., Ippel, P. F., Groff-Kellermann, B., Hoehn, H., Martin, G. M., Kubisch, C. and Oshima, J. (2010) 'WRN mutations in Werner syndrome patients: genomic rearrangements, unusual intronic mutations and ethnic-specific alterations', *Hum Genet,* 128(1), pp. 103-11.

Garaycoechea, J. I., Crossan, G. P., Langevin, F., Daly, M., Arends, M. J. and Patel, K. J. (2012) 'Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function', *Nature,* 489(7417), pp. 571-5.

Garinis, G. A., van der Horst, G. T., Vijg, J. and Hoeijmakers, J. H. (2008) 'DNA damage and ageing: new-age ideas for an age-old problem', *Nat Cell Biol,* 10(11), pp. 1241-7.

Garner, E., Kim, Y., Lach, F. P., Kottemann, M. C. and Smogorzewska, A. (2013) 'Human GEN1 and the SLX4-associated nucleases MUS81 and SLX1 are essential for the resolution of replication-induced Holliday junctions', *Cell Rep,* 5(1), pp. 207-15.

Gelot, C., Magdalou, I. and Lopez, B. S. (2015) 'Replication stress in Mammalian cells and its consequences for mitosis', *Genes (Basel),* 6(2), pp. 267-98.

German, J., Crippa, L. P. and Bloom, D. (1974) 'Bloom's syndrome. III. Analysis of the chromosome aberration characteristic of this disorder', *Chromosoma,* 48(4), pp. 361-6.

Godin, M., Francois, A., Le Roy, F., Morin, J. P., Creppy, E., Hemet, J. and Fillastre, J. P. (1996) 'Karyomegalic interstitial nephritis', *Am J Kidney Dis,* 27(1), pp. 166.

Gonzalo, S., Jaco, I., Fraga, M. F., Chen, T., Li, E., Esteller, M. and Blasco, M. A. (2006) 'DNA methyltransferases control telomere length and telomere recombination in mammalian cells', *Nat Cell Biol,* 8(4), pp. 416-24.

Gregg, S. Q., Robinson, A. R. and Niedernhofer, L. J. (2011) 'Physiological consequences of defects in ERCC1-XPF DNA repair endonuclease', *DNA Repair (Amst),* 10(7), pp. 781-91.

Halazonetis, T. D., Gorgoulis, V. G. and Bartek, J. (2008) 'An oncogene-induced DNA damage model for cancer development', *Science,* 319(5868), pp. 1352-5.

Harper, J. W. and Elledge, S. J. (2007) 'The DNA damage response: ten years after', *Mol Cell,* 28(5), pp. 739-45.

Hassen, W., Abid-Essafi, S., Achour, A., Guezzah, N., Zakhama, A., Ellouz, F., Creppy, E. E. and Bacha, H. (2004) 'Karyomegaly of tubular kidney cells in human chronic interstitial nephropathy in Tunisia: respective role of Ochratoxin A and possible genetic predisposition', *Hum Exp Toxicol,* 23(7), pp. 339-46.

Heyer, W. D. (2004) 'A new deal for Holliday junctions', *Nat Struct Mol Biol,* 11(2), pp. 117-9.

Hickson, I. D. (2003) 'RecQ helicases: caretakers of the genome', *Nat Rev Cancer,* 3(3), pp. 169-78.

Hoeijmakers, J. H. (2001) 'Genome maintenance mechanisms for preventing cancer', *Nature,* 411(6835), pp. 366-74.

Hoeijmakers, J. H. (2009) 'DNA damage, aging, and cancer', *N Engl J Med,* 361(15), pp. 1475-85.

Holm, C., Goto, T., Wang, J. C. and Botstein, D. (1985) 'DNA topoisomerase II is required at the time of mitosis in yeast', *Cell,* 41(2), pp. 553-63.

Huang, K. C., Gao, H., Yamasaki, E. F., Grabowski, D. R., Liu, S., Shen, L. L., Chan, K. K., Ganapathi, R. and Snapka, R. M. (2001) 'Topoisomerase II poisoning by ICRF-193', *J Biol Chem,* 276(48), pp. 44488-94.

Huang, S., Lee, L., Hanson, N. B., Lenaerts, C., Hoehn, H., Poot, M., Rubin, C. D., Chen, D. F., Yang, C. C., Juch, H., Dorn, T., Spiegel, R., Oral, E. A., Abid, M., Battisti, C., Lucci-Cordisco, E., Neri, G., Steed, E. H., Kidd, A., Isley, W., Showalter, D., Vittone, J. L., Konstantinow, A., Ring, J., Meyer, P., Wenger, S. L., von Herbay, A., Wollina, U., Schuelke, M., Huizenga, C. R., Leistritz, D. F., Martin, G. M., Mian, I. S. and Oshima, J. (2006) 'The spectrum of WRN mutations in Werner syndrome patients', *Hum Mutat,* 27(6), pp. 558-67.

Hustedt, N. and Durocher, D. (2016) 'The control of DNA repair by the cell cycle', *Nat Cell Biol,* 19(1), pp. 1-9.

Hwang, G., Sun, F., O'Brien, M., Eppig, J. J., Handel, M. A. and Jordan, P. W. (2017) 'SMC5/6 is required for the formation of segregation-competent bivalent chromosomes during meiosis I in mouse oocytes', *Development*.

Ira, G., Malkova, A., Liberi, G., Foiani, M. and Haber, J. E. (2003) 'Srs2 and Sgs1-Top3 suppress crossovers during double-strand break repair in yeast', *Cell,* 115(4), pp. 401-11.

Jackson, D. A. and Pombo, A. (1998) 'Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells', *J Cell Biol,* 140(6), pp. 1285-95.

Jackson, S. P. and Bartek, J. (2009) 'The DNA-damage response in human biology and disease', *Nature,* 461(7267), pp. 1071-8.

Jacome, A., Gutierrez-Martinez, P., Schiavoni, F., Tenaglia, E., Martinez, P., Rodríguez-Acebes, S., Lecona, E., Murga, M., Méndez, J., Blasco, M. A. and Fernandez-Capetillo, O. (2015) 'NSMCE2 suppresses cancer and aging in mice independently of its SUMO ligase activity', *EMBO J,* 34(21), pp. 2604-19.

Jayasurya, R., Srinivas, B. H., Ponraj, M., Haridasan, S., Parameswaran, S. and Priyamvada, P. S. (2016) 'Karyomegalic interstitial nephropathy following ifosfamide therapy', *Indian J Nephrol,* 26(4), pp. 294-7.

Jeppsson, K., Kanno, T., Shirahige, K. and Sjögren, C. (2014) 'The maintenance of chromosome structure: positioning and functioning of SMC complexes', *Nat Rev Mol Cell Biol,* 15(9), pp. 601-14.

Jeppsson, K., Carlborg, K. K., Nakato, R., Berta, D. G., Lilienthal, I., Kanno, T., Lindqvist, A., Brink, M. C., Dantuma, N. P., Katou, Y., Shirahige, K. and Sjögren, C. (2014) 'The chromosomal association of the Smc5/6 complex depends on cohesion and predicts the level of sister chromatid entanglement', *PLoS Genet,* 10(10), pp. e1004680.

Ji, Z., Zhang, L., Guo, W., McHale, C. M. and Smith, M. T. (2009) 'The benzene metabolite, hydroquinone and etoposide both induce endoreduplication in human lymphoblastoid TK6 cells', *Mutagenesis,* 24(4), pp. 367-72.

Ju, L., Wing, J., Taylor, E., Brandt, R., Slijepcevic, P., Horsch, M., Rathkolb, B., Rácz, I., Becker, L., Hans, W., Adler, T., Beckers, J., Rozman, J., Klingenspor, M., Wolf, E., Zimmer, A., Klopstock, T., Busch, D. H., Gailus-Durner, V., Fuchs, H., de Angelis, M. H., van der Horst, G. and Lehmann, A. R. (2013) 'SMC6 is an essential gene in mice, but a hypomorphic mutant in the ATPase domain has a mild phenotype with a range of subtle abnormalities', *DNA Repair (Amst),* 12(5), pp. 356-66.

Kegel, A. and Sjögren, C. (2010) 'The Smc5/6 complex: more than repair?', *Cold Spring Harb Symp Quant Biol,* 75, pp. 179-87.

Kegel, A., Betts-Lindroos, H., Kanno, T., Jeppsson, K., Ström, L., Katou, Y., Itoh, T., Shirahige, K. and Sjögren, C. (2011) 'Chromosome length influences replication-induced topological stress', *Nature,* 471(7338), pp. 392-6.

Khanna, K. K. and Jackson, S. P. (2001) 'DNA double-strand breaks: signaling, repair and the cancer connection', *Nat Genet,* 27(3), pp. 247-54.

Kliszczak, M., Stephan, A. K., Flanagan, A. M. and Morrison, C. G. (2012) 'SUMO ligase activity of vertebrate Mms21/Nse2 is required for efficient DNA repair but not for Smc5/6 complex stability', *DNA Repair (Amst),* 11(10), pp. 799-810.

Kottemann, M. C. and Smogorzewska, A. (2013) 'Fanconi anaemia and the repair of Watson and Crick DNA crosslinks', *Nature,* 493(7432), pp. 356-63.

Kudlow, B. A., Kennedy, B. K. and Monnat, R. J. (2007) 'Werner and Hutchinson-Gilford progeria syndromes: mechanistic basis of human progeroid diseases', *Nat Rev Mol Cell Biol,* 8(5), pp. 394-404.

Lachaud, C., Castor, D., Hain, K., Muñoz, I., Wilson, J., MacArtney, T. J., Schindler, D. and Rouse, J. (2014) 'Distinct functional roles for the two SLX4 ubiquitin-binding UBZ domains mutated in Fanconi anemia', *J Cell Sci,* 127(Pt 13), pp. 2811-7.

Lachaud, C., Slean, M., Marchesi, F., Lock, C., Odell, E., Castor, D., Toth, R. and Rouse, J. (2016) 'Karyomegalic interstitial nephritis and DNA damage-induced polyploidy in Fan1 nuclease-defective knock-in mice', *Genes Dev,* 30(6), pp. 639-44.

Langevin, F., Crossan, G. P., Rosado, I. V., Arends, M. J. and Patel, K. J. (2011) 'Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice', *Nature,* 475(7354), pp. 53-8.

Lans, H. and Hoeijmakers, J. H. (2012) 'Genome stability, progressive kidney failure and aging', *Nat Genet,* 44(8), pp. 836-8.

Lecona, E., Barrasa, J. I., Olmo, N., Llorente, B., Turnay, J. and Lizarbe, M. A. (2008) 'Upregulation of annexin A1 expression by butyrate in human colon adenocarcinoma cells: role of p53, NF-Y, and p38 mitogen-activated protein kinase', *Mol Cell Biol,* 28(15), pp. 4665-74.

Lecona, E. and Fernández-Capetillo, O. (2014) 'Replication stress and cancer: it takes two to tango', *Exp Cell Res,* 329(1), pp. 26-34.

Lehmann, A. R., Walicka, M., Griffiths, D. J., Murray, J. M., Watts, F. Z., McCready, S. and Carr, A. M. (1995) 'The rad18 gene of Schizosaccharomyces pombe defines a new subgroup of the SMC superfamily involved in DNA repair', *Mol Cell Biol,* 15(12), pp. 7067-80.

Liberi, G., Maffioletti, G., Lucca, C., Chiolo, I., Baryshnikova, A., Cotta-Ramusino, C., Lopes, M., Pellicioli, A., Haber, J. E. and Foiani, M. (2005) 'Rad51-dependent DNA structures accumulate at damaged replication forks in sgs1 mutants defective in the yeast ortholog of BLM RecQ helicase', *Genes Dev,* 19(3), pp. 339-50.

Lindahl, T. (1993) 'Instability and decay of the primary structure of DNA', *Nature,* 362(6422), pp. 709-15.

Lindroos, H. B., Ström, L., Itoh, T., Katou, Y., Shirahige, K. and Sjögren, C. (2006) 'Chromosomal association of the Smc5/6 complex reveals that it functions in differently regulated pathways', *Mol Cell,* 22(6), pp. 755-67.

Liu, Y. and West, S. C. (2004) 'Happy Hollidays: 40th anniversary of the Holliday junction', *Nat Rev Mol Cell Biol,* 5(11), pp. 937-44.

Lobitz, S. and Velleuer, E. (2006) 'Guido Fanconi (1892-1979): a jack of all trades', *Nat Rev Cancer,* 6(11), pp. 893-8.

Loonstra, A., Vooijs, M., Beverloo, H. B., Allak, B. A., van Drunen, E., Kanaar, R., Berns, A. and Jonkers, J. (2001) 'Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells', *Proc Natl Acad Sci U S A,* 98(16), pp. 9209-14.

Losada, A. and Hirano, T. (2005) 'Dynamic molecular linkers of the genome: the first decade of SMC proteins', *Genes Dev,* 19(11), pp. 1269-87.

Lukas, C., Savic, V., Bekker-Jensen, S., Doil, C., Neumann, B., Pedersen, R. S., Grøfte, M., Chan, K. L., Hickson, I. D., Bartek, J. and Lukas, J. (2011) '53BP1 nuclear bodies form around DNA lesions generated by mitotic transmission of chromosomes under replication stress', *Nat Cell Biol,* 13(3), pp. 243-53.

López-Contreras, A. J. and Fernandez-Capetillo, O. (2010) 'The ATR barrier to replication-born DNA damage', *DNA Repair (Amst),* 9(12), pp. 1249-55.

MacKay, C., Déclais, A. C., Lundin, C., Agostinho, A., Deans, A. J., MacArtney, T. J., Hofmann, K., Gartner, A., West, S. C., Helleday, T., Lilley, D. M. and Rouse, J. (2010) 'Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2', *Cell,* 142(1), pp. 65-76.

Magdalou, I., Lopez, B. S., Pasero, P. and Lambert, S. A. (2014) 'The causes of replication stress and their consequences on genome stability and cell fate', *Semin Cell Dev Biol,* 30, pp. 154-64.

Mally, A. (2012) 'Ochratoxin a and mitotic disruption: mode of action analysis of renal tumor formation by ochratoxin A', *Toxicol Sci,* 127(2), pp. 315-30.

Mally, A., Pepe, G., Ravoori, S., Fiore, M., Gupta, R. C., Dekant, W. and Mosesso, P. (2005) 'Ochratoxin a causes DNA damage and cytogenetic effects but no DNA adducts in rats', *Chem Res Toxicol,* 18(8), pp. 1253-61.

Mankouri, H. W., Ashton, T. M. and Hickson, I. D. (2011) 'Holliday junction-containing DNA structures persist in cells lacking Sgs1 or Top3 following exposure to DNA damage', *Proc Natl Acad Sci U S A,* 108(12), pp. 4944-9.

Mankouri, H. W., Huttner, D. and Hickson, I. D. (2013) 'How unfinished business from S-phase affects mitosis and beyond', *EMBO J,* 32(20), pp. 2661-71.

Manthei, K. A. and Keck, J. L. (2013) 'The BLM dissolvasome in DNA replication and repair', *Cell Mol Life Sci,* 70(21), pp. 4067-84.

Maslov, A. Y. and Vijg, J. (2009) 'Genome instability, cancer and aging', *Biochim Biophys Acta,* 1790(10), pp. 963-9.

Matos, J. and West, S. C. (2014) 'Holliday junction resolution: regulation in space and time', *DNA Repair (Amst),* 19, pp. 176-81.

McCulloch, T., Prayle, A., Lunn, A. and Watson, A. R. (2011) 'Karyomegalic-like nephropathy, Ewing's sarcoma and ifosfamide therapy', *Pediatr Nephrol,* 26(7), pp. 1163-6.

McDonald, W. H., Pavlova, Y., Yates, J. R. and Boddy, M. N. (2003) 'Novel essential DNA repair proteins Nse1 and Nse2 are subunits of the fission yeast Smc5-Smc6 complex', *J Biol Chem,* 278(46), pp. 45460-7.

McWhir, J., Selfridge, J., Harrison, D. J., Squires, S. and Melton, D. W. (1993) 'Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning', *Nat Genet,* 5(3), pp. 217-24.

Menolfi, D., Delamarre, A., Lengronne, A., Pasero, P. and Branzei, D. (2015) 'Essential Roles of the Smc5/6 Complex in Replication through Natural Pausing Sites and Endogenous DNA Damage Tolerance', *Mol Cell,* 60(6), pp. 835-46.

Michl, J., Zimmer, J. and Tarsounas, M. (2016) 'Interplay between Fanconi anemia and homologous recombination pathways in genome integrity', *EMBO J,* 35(9), pp. 909-23.

Mihatsch, M. J., Gudat, F., Zollinger, H. U., Heierli, C., Thölen, H. and Reutter, F. W. (1979) 'Systemic karyomegaly associated with chronic interstitial nephritis. A new disease entity?', *Clin Nephrol,* 12(2), pp. 54-62.

Mimitou, E. P. and Symington, L. S. (2009) 'Nucleases and helicases take center stage in homologous recombination', *Trends Biochem Sci,* 34(5), pp. 264-72.

Moldovan, G. L. and D'Andrea, A. D. (2009) 'How the fanconi anemia pathway guards the genome', *Annu Rev Genet,* 43, pp. 223-49.

Morgado-Palacin, I., Day, A., Murga, M., Lafarga, V., Anton, M. E., Tubbs, A., Chen, H. T., Ergan, A., Anderson, R., Bhandoola, A., Pike, K. G., Barlaam, B., Cadogan, E., Wang, X., Pierce, A. J., Hubbard, C., Armstrong, S. A., Nussenzweig, A. and Fernandez-Capetillo, O. (2016) 'Targeting the kinase activities of ATR and ATM exhibits antitumoral activity in mouse models of MLL-rearranged AML', *Sci Signal,* 9(445), pp. ra91.

Mosesso, P., Cinelli, S., Piñero, J., Bellacima, R. and Pepe, G. (2008) 'In vitro cytogenetic results supporting a DNA nonreactive mechanism for ochratoxin A, potentially relevant for its carcinogenicity', *Chem Res Toxicol,* 21(6), pp. 1235-43.

Mourón, S., Rodriguez-Acebes, S., Martínez-Jiménez, M. I., García-Gómez, S., Chocrón, S., Blanco, L. and Méndez, J. (2013) 'Repriming of DNA synthesis at stalled replication forks by human PrimPol', *Nat Struct Mol Biol,* 20(12), pp. 1383-9.

Murga, M., Bunting, S., Montaña, M. F., Soria, R., Mulero, F., Cañamero, M., Lee, Y., McKinnon, P. J., Nussenzweig, A. and Fernandez-Capetillo, O. (2009) 'A mouse model of

ATR-Seckel shows embryonic replicative stress and accelerated aging', *Nat Genet,* 41(8), pp. 891-8.

Murga, M., Campaner, S., Lopez-Contreras, A. J., Toledo, L. I., Soria, R., Montaña, M. F., D'Artista, L., Schleker, T., Guerra, C., Garcia, E., Barbacid, M., Hidalgo, M., Amati, B. and Fernandez-Capetillo, O. (2011) 'Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors', *Nat Struct Mol Biol,* 18(12), pp. 1331-5.

Murray, J. M. and Carr, A. M. (2008) 'Smc5/6: a link between DNA repair and unidirectional replication?', *Nat Rev Mol Cell Biol,* 9(2), pp. 177-82.

Nasim, A. and Smith, B. P. (1975) 'Genetic control of radiation sensitivity in Schizosaccharomyces pombe', *Genetics,* 79(4), pp. 573-82.

Nieto-Soler, M., Morgado-Palacin, I., Lafarga, V., Lecona, E., Murga, M., Callen, E., Azorin, D., Alonso, J., Lopez-Contreras, A. J., Nussenzweig, A. and Fernandez-Capetillo, O. (2016) 'Efficacy of ATR inhibitors as single agents in Ewing sarcoma', *Oncotarget,* 7(37), pp. 58759-58767.

Nitiss, J. L. (2009) 'DNA topoisomerase II and its growing repertoire of biological functions', *Nat Rev Cancer,* 9(5), pp. 327-37.

O'Driscoll, M., Ruiz-Perez, V. L., Woods, C. G., Jeggo, P. A. and Goodship, J. A. (2003) 'A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome', *Nat Genet,* 33(4), pp. 497-501.

Outwin, E. A., Irmisch, A., Murray, J. M. and O'Connell, M. J. (2009) 'Smc5-Smc6-dependent removal of cohesin from mitotic chromosomes', *Mol Cell Biol,* 29(16), pp. 4363-75.

Palecek, J. J. and Gruber, S. (2015) 'Kite Proteins: a Superfamily of SMC/Kleisin Partners Conserved Across Bacteria, Archaea, and Eukaryotes', *Structure,* 23(12), pp. 2183-90.

Payne, F., Colnaghi, R., Rocha, N., Seth, A., Harris, J., Carpenter, G., Bottomley, W. E., Wheeler, E., Wong, S., Saudek, V., Savage, D., O'Rahilly, S., Carel, J. C., Barroso, I., O'Driscoll, M. and Semple, R. (2014) 'Hypomorphism in human NSMCE2 linked to primordial dwarfism and insulin resistance', *J Clin Invest,* 124(9), pp. 4028-38.

Payne, F., Colnaghi, R., Rocha, N., Seth, A., Harris, J., Carpenter, G., Bottomley, W. E., Wheeler, E., Wong, S., Saudek, V., Savage, D., O'Rahilly, S., Carel, J. C., Barroso, I., O'Driscoll, M. and Semple, R. (2014) 'Hypomorphism in human NSMCE2 linked to primordial dwarfism and insulin resistance', *J Clin Invest,* 124(9), pp. 4028-38.

Pebernard, S., Perry, J. J., Tainer, J. A. and Boddy, M. N. (2008) 'Nse1 RING-like domain supports functions of the Smc5-Smc6 holocomplex in genome stability', *Mol Biol Cell,* 19(10), pp. 4099-109.

Pebernard, S., Schaffer, L., Campbell, D., Head, S. R. and Boddy, M. N. (2008b) 'Localization of Smc5/6 to centromeres and telomeres requires heterochromatin and SUMO, respectively', *EMBO J,* 27(22), pp. 3011-23.

Pontel, L. B., Rosado, I. V., Burgos-Barragan, G., Garaycoechea, J. I., Yu, R., Arends, M. J., Chandrasekaran, G., Broecker, V., Wei, W., Liu, L., Swenberg, J. A., Crossan, G. P. and Patel, K. J. (2015) 'Endogenous Formaldehyde Is a Hematopoietic Stem Cell Genotoxin and Metabolic Carcinogen', *Mol Cell,* 60(1), pp. 177-88.

Prakash, S. and Prakash, L. (1977) 'Increased spontaneous mitotic segregation in MMS-sensitive mutants of Saccharomyces cerevisiae', *Genetics,* 87(2), pp. 229-36.

Rattner, J. B., Hendzel, M. J., Furbee, C. S., Muller, M. T. and Bazett-Jones, D. P. (1996) 'Topoisomerase II alpha is associated with the mammalian centromere in a cell cycle- and species-specific manner and is required for proper centromere/kinetochore structure', *J Cell Biol,* 134(5), pp. 1097-107.

Rosado, I. V., Langevin, F., Crossan, G. P., Takata, M. and Patel, K. J. (2011) 'Formaldehyde catabolism is essential in cells deficient for the Fanconi anemia DNA-repair pathway', *Nat Struct Mol Biol,* 18(12), pp. 1432-4.

Rossi, M. L., Ghosh, A. K. and Bohr, V. A. (2010) 'Roles of Werner syndrome protein in protection of genome integrity', *DNA Repair (Amst),* 9(3), pp. 331-44.

Ruiz, S., Panopoulos, A. D., Herrerías, A., Bissig, K. D., Lutz, M., Berggren, W. T., Verma, I. M. and Izpisua Belmonte, J. C. (2011) 'A high proliferation rate is required for cell reprogramming and maintenance of human embryonic stem cell identity', *Curr Biol,* 21(1), pp. 45-52.

Ruiz, S., Mayor-Ruiz, C., Lafarga, V., Murga, M., Vega-Sendino, M., Ortega, S. and Fernandez-Capetillo, O. (2016) 'A Genome-wide CRISPR Screen Identifies CDC25A as a Determinant of Sensitivity to ATR Inhibitors', *Mol Cell,* 62(2), pp. 307-13.

Ruzankina, Y., Pinzon-Guzman, C., Asare, A., Ong, T., Pontano, L., Cotsarelis, G., Zediak, V. P., Velez, M., Bhandoola, A. and Brown, E. J. (2007) 'Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss', *Cell Stem Cell,* 1(1), pp. 113-26.

Seckel, H., Bird-Headed Dwarfs: Studies in Developmental Anthropology Including Human Proportions (Karger, Basel, Switzerland, 1960).

Shrivastav, M., De Haro, L. P. and Nickoloff, J. A. (2008) 'Regulation of DNA double-strand break repair pathway choice', *Cell Res,* 18(1), pp. 134-47.

Smogorzewska, A., Desetty, R., Saito, T. T., Schlabach, M., Lach, F. P., Sowa, M. E., Clark, A. B., Kunkel, T. A., Harper, J. W., Colaiácovo, M. P. and Elledge, S. J. (2010) 'A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair', *Mol Cell,* 39(1), pp. 36-47.

Sollier, J., Driscoll, R., Castellucci, F., Foiani, M., Jackson, S. P. and Branzei, D. (2009) 'The Saccharomyces cerevisiae Esc2 and Smc5-6 proteins promote sister chromatid junction-mediated intra-S repair', *Mol Biol Cell,* 20(6), pp. 1671-82

Stephan, A. K., Kliszczak, M. and Morrison, C. G. (2011) 'The Nse2/Mms21 SUMO ligase of the Smc5/6 complex in the maintenance of genome stability', *FEBS Lett,* 585(18), pp. 2907-13.

Stingele, J., Bellelli, R., Alte, F., Hewitt, G., Sarek, G., Maslen, S. L., Tsutakawa, S. E., Borg, A., Kjær, S., Tainer, J. A., Skehel, J. M., Groll, M. and Boulton, S. J. (2016) 'Mechanism and Regulation of DNA-Protein Crosslink Repair by the DNA-Dependent Metalloprotease SPRTN', *Mol Cell,* 64(4), pp. 688-703.

Storchova, Z. and Pellman, D. (2004) 'From polyploidy to aneuploidy, genome instability and cancer', *Nat Rev Mol Cell Biol,* 5(1), pp. 45-54.

Sumner, A. T. (1998) 'Induction of diplochromosomes in mammalian cells by inhibitors of topoisomerase II', *Chromosoma,* 107(6-7), pp. 486-90.

Swuec, P. and Costa, A. (2014) 'Molecular mechanism of double Holliday junction dissolution', *Cell Biosci,* 4, pp. 36.

Takahashi, Y., Dulev, S., Liu, X., Hiller, N. J., Zhao, X. and Strunnikov, A. (2008) 'Cooperation of sumoylated chromosomal proteins in rDNA maintenance', *PLoS Genet,* 4(10), pp. e1000215.

Taniai, E., Yafune, A., Nakajima, M., Hayashi, S. M., Nakane, F., Itahashi, M. and Shibutani, M. (2014) 'Ochratoxin A induces karyomegaly and cell cycle aberrations in renal tubular cells without relation to induction of oxidative stress responses in rats', *Toxicol Lett,* 224(1), pp. 64-72.

Thompson, B. J., Escarmis, C., Parker, B., Slater, W. C., Doniger, J., Tessman, I. and Warner, R. C. (1975) 'Figure-8 configuration of dimers of S13 and phiX174 replicative form DNA', *J Mol Biol,* 91(4), pp. 409-19.

Thongthip, S., Bellani, M., Gregg, S. Q., Sridhar, S., Conti, B. A., Chen, Y., Seidman, M. M. and Smogorzewska, A. (2016) 'Fan1 deficiency results in DNA interstrand cross-link repair defects, enhanced tissue karyomegaly, and organ dysfunction', *Genes Dev,* 30(6), pp. 645-59.

Trujillo, J. P., Mina, L. B., Pujol, R., Bogliolo, M., Andrieux, J., Holder, M., Schuster, B., Schindler, D. and Surrallés, J. (2012) 'On the role of FAN1 in Fanconi anemia', *Blood,* 120(1), pp. 86-9.

Torres-Rosell, J., Machín, F., Farmer, S., Jarmuz, A., Eydmann, T., Dalgaard, J. Z. and Aragón, L. (2005) 'SMC5 and SMC6 genes are required for the segregation of repetitive chromosome regions', *Nat Cell Biol,* 7(4), pp. 412-9.

Uemura, T. and Yanagida, M. (1984) 'Isolation of type I and II DNA topoisomerase mutants from fission yeast: single and double mutants show different phenotypes in cell growth and chromatin organization', *EMBO J,* 3(8), pp. 1737-44.

van der Crabben, S. N., Hennus, M. P., McGregor, G. A., Ritter, D. I., Nagamani, S. C., Wells, O. S., Harakalova, M., Chinn, I. K., Alt, A., Vondrova, L., Hochstenbach, R., van

Montfrans, J. M., Terheggen-Lagro, S. W., van Lieshout, S., van Roosmalen, M. J., Renkens, I., Duran, K., Nijman, I. J., Kloosterman, W. P., Hennekam, E., Orange, J. S., van Hasselt, P. M., Wheeler, D. A., Palecek, J. J., Lehmann, A. R., Oliver, A. W., Pearl, L. H., Plon, S. E., Murray, J. M. and van Haaften, G. (2016) 'Destabilized SMC5/6 complex leads to chromosome breakage syndrome with severe lung disease', *J Clin Invest,* 126(8), pp. 2881-92.

Verver, D. E., Hwang, G. H., Jordan, P. W. and Hamer, G. (2016) 'Resolving complex chromosome structures during meiosis: versatile deployment of Smc5/6', *Chromosoma,* 125(1), pp. 15-27.

Verver, D. E., Zheng, Y., Speijer, D., Hoebe, R., Dekker, H. L., Repping, S., Stap, J. and Hamer, G. (2016) 'Non-SMC Element 2 (NSMCE2) of the SMC5/6 Complex Helps to Resolve Topological Stress', *Int J Mol Sci,* 17(11).

Vettorazzi, A., de Trocóniz, I. F., González-Peñas, E., Arbillaga, L., Corcuera, L. A., Gil, A. G. and de Cerain, A. L. (2011) 'Kidney and liver distribution of ochratoxin A in male and female F344 rats', *Food Chem Toxicol,* 49(9), pp. 1935-42.

Wang, W. (2008) 'A major switch for the Fanconi anemia DNA damage-response pathway', *Nat Struct Mol Biol,* 15(11), pp. 1128-30.

Weeda, G., Donker, I., de Wit, J., Morreau, H., Janssens, R., Vissers, C. J., Nigg, A., van Steeg, H., Bootsma, D. and Hoeijmakers, J. H. (1997) 'Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence', *Curr Biol,* 7(6), pp. 427-39.

West, S. C., Blanco, M. G., Chan, Y. W., Matos, J., Sarbajna, S. and Wyatt, H. D. (2015) 'Resolution of Recombination Intermediates: Mechanisms and Regulation', *Cold Spring Harb Symp Quant Biol,* 80, pp. 103-9.

Wyatt, H. D., Sarbajna, S., Matos, J. and West, S. C. (2013) 'Coordinated actions of SLX1-SLX4 and MUS81-EME1 for Holliday junction resolution in human cells', *Mol Cell,* 52(2), pp. 234-47.

Xaver, M., Huang, L., Chen, D. and Klein, F. (2013) 'Smc5/6-mms21 prevents and eliminates inappropriate recombination intermediates in meiosis', *PLoS Genet,* 9(12), pp. e1004067.

Yong-Gonzales, V., Hang, L. E., Castellucci, F., Branzei, D. and Zhao, X. (2012) 'The Smc5-Smc6 complex regulates recombination at centromeric regions and affects kinetochore protein sumoylation during normal growth', *PLoS One,* 7(12), pp. e51540.

Zhou, W., Otto, E. A., Cluckey, A., Airik, R., Hurd, T. W., Chaki, M., Diaz, K., Lach, F. P., Bennett, G. R., Gee, H. Y., Ghosh, A. K., Natarajan, S., Thongthip, S., Veturi, U., Allen, S. J., Janssen, S., Ramaswami, G., Dixon, J., Burkhalter, F., Spoendlin, M., Moch, H., Mihatsch, M. J., Verine, J., Reade, R., Soliman, H., Godin, M., Kiss, D., Monga, G., Mazzucco, G., Amann, K., Artunc, F., Newland, R. C., Wiech, T., Zschiedrich, S., Huber, T. B., Friedl, A., Slaats, G. G., Joles, J. A., Goldschmeding, R., Washburn, J., Giles, R. H., Levy, S., Smogorzewska, A. and Hildebrandt, F. (2012) 'FAN1 mutations cause karyomegalic

interstitial nephritis, linking chronic kidney failure to defective DNA damage repair', *Nat Genet,* 44(8), pp. 910-5.